Yale iGEM

Lab Notebook 2015

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WEEK -1 (Before 6/1, 2015)

Note: Dan Shapiro conducted all Week -1 research

5/19/2015

- Had an orientaton! How-to's of electroporation, growing cultures, introduction to the lab, etc.
- **Cyano:**
  - Found *nirA* gene with promoter on biocyc.org.
    - Went 170 bp upstream to find promoter, took idea from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1265929/
    - "A 166-bp fragment upstream of the *nirA* operon was amplified by PCR from the genomic DNA of *Synechococcus* sp. strain PCC 7942"
    - "The amplified region possesses an NtcB-binding site (an inverted repeat with a LysR motif, TGCAN5TGCA)"
    - "This DNA segment has been showed to retain full responsiveness to nitrogen and transcription activity"
- **Bio-pigment**
  - Started a culture of E. Coli with SDS resistance
  - Found and isolated 7 bio-pigment biobricks

Part	Genes	Product	URL	Year	Plate	Well	Resistance
BBa_K274100	CrtEBI	Lycopene	http://parts.igem.org/partsdb/get_part.cgi?id=14947&show=1	2012	3	6H	Amp
BBa_K274111	CrtY w/ pBAD	Beta-carotene	http://parts.igem.org/Part:BBa_K274111	2012	4	18E	Kan
BBa_K274110	CrtEBI under constitutive promoter	Lycopene	http://parts.igem.org/Part:BBa_K274110	2012	3	6J	Amp
BBa_K274002	VioABCDE	Violacein	http://parts.igem.org/Part:BBa_K274002	2012	4	20A	tetR
BBa_K274004	VioABCE	Violacein (light green)	http://parts.igem.org/Part:BBa_K274004	2012	4	18C	Kan
BBa_K274003	VioABDE	Violacein (green)	http://parts.igem.org/Part:BBa_K274003	2012	4	18A	Kan
BBa_K274200	CrtEBIY	Beta-carotene	http://parts.igem.org/Part:BBa_K274200	2012	3	6L	Amp

5/20/2015

- **Cyano:**
  - Found psbA2 light-inducible promoter
    - Sequence is in table on page 2 of http://ac.els-cdn.com/S1522472400902277/1-s2.0-S1522472400902277-main.pdf?_tid=2c2a3304-ff23-11e4-a38b-00000aacb35d&acdnat=1432148905_37bf8f4f11026bbd33007a42b36d239f
  - Found isiAB iron-dependent promoter, works 5000-fold!

        - Fur-box works by restricting translation of protein, not transcription of mRNA, however.
        - http://femsle.oxfordjournals.org/content/227/2/255.long#T2
    - Also found and isolated backbone plasmid on the iGEM parts registry
        - ttp://parts.igem.org/Part:BBa_K125000
        - Low-copy number, broad-host-range vector
- **Bio-Pigment:**
    - Transformed pigment bio bricks into E. Coli strain as such:

#	DNA	Time constant (TC)	Plate on
T1	Bba_K274100	5.1	T1 Carb
T2	Bba_K274111	5.0	T2 Kan
T3	Bba_K274110	4.9	T3 Carb
T4	Bba_K274002	4.9	T4 CamR
T5	Bba_K274004	4.9	T5 CamR
T6	Bba_K274003	4.8	T6 CamR
T7	Bba_K2742000	5.1	T7 Carb
T8	**Bba_K125000**	**5.1**	**T8 Spec**

    - NOTE: **T8** is for the Cyanobacteria project

5/21/2015

- **Cyano:**
    - Nothing of importance
- **Bio-pigment production**
    - T1,2,3,7,8 worked, cultures were made
        - Frozen stock of T1,2,3,7,8 made
    - T4,5,6 *failed twice*, because we're fools and put them on the wrong antibiotics twice.
    - T4,5,6 redone with correct antibiotic (CamR) - All turned purple prior to electroporation when mixed with violacein DNA. This was strange, and indicated possible contamination

5/26/2015

- **Cyano:**
    - Prepared 500 mL of the E. Coli with the plasmid backbone (Bba_K125000) for a Maxi Prep Tomorrow.
    - Realized that the Synechococcus 6803 Cyanobacteria were drying out and dying, so we moistened them with distilled water and began preparing media.
- **Bio-pigment production**
    - For some reason, while T1,4,5, and 6 grew normally, *so did the controls!* This should not have happened - this jeopardizes all the plates used in this step, as well as the frozen stocks, since the plates may have been faulty. 48 wells of a 96-well plate were filled with media and the relevant antibiotic, and T1,4,5,6, and controls were tested to see if the plates failed or they actually got the plasmid needed. Results coming tomorrow.

5/27/2015

- **Cyano:**
  - Scraped out a few cells from the totally dried out Synechococcus 6803 sample, and made a template using the cells and distilled water.
  - Made primers to isolate the *nirA* and *isiAB* promoters, and created primers which contained Anderson promoter BBa_J23100 and annealing portions to Green Fluorescent Protein (GFP). The GFP would be used to check whether or not the promoters worked.
  - Used this PCR protocol to attempt and isolate the promoters:
    - ALL: 20 uL polymerase mix, .5 uL forward primer, .5 uL reverse primer, 2 uL backbone,17 uL qH_2O.
    - 5 min 95C
    - 15 sec 98C
    - 15 sec 58C
    - 30 sec 72C
    - Repeat b-c-d 30x
    - 5 min 72C
    - Indefinite 12C

- Did a Maxi-Prep with the 500 mL of E. Coli with the Bba_K125000 plasmid backbone in them.
  - Concentration resulting: 9.6 ng/mL
  - 280/260 ratio: 1.88
  - Followed the Qaigen Maxi-Prep procedure.
- **Bio-Pigment:**
  - Everything grew in the 96 well plate from yesterday (T1, 4,5,6, and controls) so it is clear that the antibiotic failed since the bacteria shouldn't have resistance to both Carb and Chloro. Could be something with the strain?
  - Everything frozen, new strain will probably be used later.
  - Miniprep to be done tomorrow to assess which strains T1,4,5,6 have the plasmid we want, if at all.

5/28/2015

- **Cyano:**
  - Ran the first PCR of the nirA and isiAB promoters, GFP DNA fragment, and Anderson promoter-GFPs. Didn't work very well, and neither did the ladder. Gel got thrown away.
  - Ran a PCR screen of the plasmids from the maxi prep to see if they worked and if we have any Plasmid. Also re-ran the nirA, isiAB, GFP, and Andsn GFP DNA plus primers.
  - After Gel Purification, only GFP, Andsn GFP, and isiAB worked, but barely
    - GFP: 8.2 ng/mL
    - isiAB: 6.7 ng/mL
    - Andsn GFP: 27.5 ng/mL
- **Bio-Pigment:**
  - We decided that the NJM 795 strain that we were using isn't all that good; turns out Natalie has had trouble with it in the past with weird mutations and baseline Carbenicillin resistance. A new culture was made today using the NJM

150 (MG 16550), which is a no-frills strain of E. Coli that is the ancestor strain in the lab. A frozen stock will be made tomorrow, and new transformations will be done with all the biobrick pigments to make sure they actually work.

5/29/2015

- **Cyano:**
  - Gels were run again with the GFP and cyanobacteria primers.
    - isiAB had a concentration of 4.642 ng/uL
    - GFP had a concentration of 7.418 ng/uL
    - The Anderson Promoter + GFP DNA had a concentration of 12.882 ng/uL
  - We should probably amplify the cyanobacteria genomes to get a better concentration.
- **Bio-Pigment:**
  - Transformations didn't work, as proved by PCR. T2,3,7,8 all had no DNA. With how the Cyanobacteria project was progressing, with some results, it was clear the bio-pigment project had to be scrapped.

WEEK 1 (6/1 to 6/7, 2015)

MONDAY, 6/1

- Orientation!
    - Graduate mentors showed us around the lab space
    - We ran a practice transformation with GFP and BFP genes in E. coli-compatible plasmids
    - Screening of *Meet the Robinsons* to learn about failure in research
    - Meetings with faculty advisors

TUESDAY, 6/2

- Organizing our lab space
- Group meeting
    - Graduate mentors pointed us towards some papers and resources that could give us an idea of the viability of porting MAGE into cyanobacterium and rhizobium
    - Logistics and communications issues were addressed as well

WEDNESDAY, 6/3

- Meeting: Reaearch Planning
    - Beta homologs will take time to arrive (2ish weeks)
        - So, in the meantime, we need to figure out other conditions:
            - Growth conditions
            - Plasmids
    - Lab Techniques and Time
        - Ordering gblocks/dsDNA: 1-2 weeks
        - PCR: a few hours
        - Cloning (entire process, from fragments to verified plasmid): 3-4 days (or 2 years)
            - PCR, Gel Extraction, Gibson Assembly, Transformation, submit for sequencing
        - MAGE cycles: 2-3 days per cycle
    - Knock out *mutS*
        - MutS recognizes and marks DNA mismatches for repair, and thus hinders the efficiency of MAGE. It should be knocked out in our organisms to increase mutagenesis efficiency.
        - How to knock out MutS is a bit of a problem… multiple options
            - Flip recombinase (Eddie)
            - Or use the CRISPR and Cas9 from biobricks
    - We should get cyano to test lab techniques and transformation
    - Moving Forward:
        - Have beta-homologs ordered by the end of week two
        - **Dan** will synthesize a week-by-week research plan
        - **Danny** and **Erin** will keep looking into beta-homologs
        - **Colin** will look into knocking out MutS gene
        - **Dan** is the promoter guy

THURSDAY, 6/4

- Meeting with Jaymin (graduate mentor)
  - We could have a chance to visit Peccia lab (cyanobacteria culturing) next week
    - See layout
    - Get growth/storage protocols
    - Ask about 7002's natural competency
    - Sometime next Monday (6/8?)
  - Organizing Beta-Homolog Constructs
    - Promoters
    - Vectors
    - Backbones
    - Beta-proteins
  - Draw out all sequences *in silico* so that they are ready to order when needed
  - Jaymin will e-mail Delaporta to get sequences of citrine (*mcit*)
  - CYANO CONSTRUCTS
    - Plasmid:
      - BBa_K125000 (has spectinomycin and streptomycin resistance)
      - (low copy number, should work in e. coli and cyano)
    - Promoters:
      - Inducible:
        - psaA
        - nirA
        - isiAB
        - Green Light Inducible
        - tetR
        - pBAD
        - Lambda-CI
      - Constitutive:
        - Anderson promoters (strong, medium, weak), pulled from Parts Registry
      - Citrine gene
        - (Jaymin will get sequence from Delaporta and pass them on to us
      - **BIG GOAL: By the end of the day, have primers designed for all plasmids with combinations of promoters**
        - Guidelines:
          - Homology regions are ~35 bp
          - Forward and reverse primers of plasmid will remain constant
          - Forward primer for citrine will remain constant
          - Reverse compliment primer for plasmid to citrine (w/ homology) will remain constant
          - Forward primer for plasmid to promoter (homology on promoter) will CHANGE
          - Reverse compliment primer from citrine to end of promoter (homology on citrine) will CHANGE

- Workflow for beginning of summer:
  - Day 1: Order primer
  - Day 2: PCR fragments
  - Day 3: Cloning (Gibson assembly), transform into e. coli
  - Day 4: Screen colonies, submit for sequencing (by 4 PM)
  - Day 5: Obtain sequencing results
  - Day 6-15: Test promoters in cyano

- **Moving forward:**
  i. Finalize list of beta-homologs, obtain sequences
  ii. Finalize list of promoters and characterize them as much as possible in silico
  iii. Design primers for K125000/promoters/citrine
  iv. Do steps 1-3 for rhizo
  v. Find reliable inducible promoters
  vi. Adapt FLP recombinase protocol to knockout MutS (CFH will take lead on this)

- When 7002 arrives:
  - Do FLP recombinase to knockout *mutS*
  - Perform a baseline MAGE with electroporation
    - Rifampicin Assay – This could help to characterize the natural recombinogenecy of the cyanobacteria
    - SSb protein in almost all bacteria might be responsible for the natural recombinogenecy, but overexpressing it in cyano may not help with the MAGE process (as it doesn't in e. coli)

FRIDAY, 6/5

- Holly
  - Goal: design primers for chosen vectors and gene (GFP)
  - Purpose: the plasmid will be inserted into E. coli to test whether the promoter & plasmid combination works, before insertion of beta-homologs
  - Chosen vectors: pMK2016, pRm1132f (sequences found on NCBI
  - Gene: sfGFP_K12 (on Google Drive)
- The rest of the team helped Holly with designing primers and continued with online literature searches

WEEK 2 (6/8 to 6/14, 2015)

6/08/2015

Meeting with PIs

- Things to look into:
  - Natural recombinogenecy of cyano
  - An experiment to characterize natural competency
  - Past transformation methods for cyano (someone should acquire the failed protocols from the Peccia lab)
  - Compare Peccia lab cyano transformation methods with those described in the FLP cyano paper
  - Has the rifampicin assay been done specifically in cyano?
  - Other gene modification reporting assays that are well established in cyano:
    - Photosynthetic mutations
    - nbla gene knockout (Ed mentioned this, turns green cells into albinos)
  - An easily-presentable project for iGEM:
    - Production of indigo?
  - A GENERALIZABLE STRATEGY: designing vectors with a broad host range including E. coli, PCC 7002, and S. meliloti
  - Dellaporta might have good information on a quick, efficient assembly system (non-Gibson)
- Knocking out mutS:
  - Colin will keep looking into FLP system
    - Main issue: finding plasmids
    - Why not just stick in linear DNA instead of a plasmid to incorporate FRT sites and kan^r cassette?
  - Danny will look into CRISPR
- TEAM TASKS:

Task	Cyano	Rhizo
Finding Beta-Homologs	DJK	JT
Delivery Methods	CFH	HJZ/EYW
Vectors and Promotors	DS	LJ
Feasible Assay	To Be Determined	To Be Determined
Culturing	EYW	HJZ

6/09/2015

- Cyanobacteria
  - Fresh cultures of synechocystis sp. 6803 were prepared in BG-11
  - Fresh cultures of UTEX 2973 were prepared in BG-11
  - Literature search to determine best practice protocols.

6/10/2015

- Rhizobium
  - Obtained two strains of Rhizobium from the Handelsman Lab (Rhizobium tropici CIAT 899 and Rhizobium etli CE3)
  - Made Tryptic Soy Broth medium (growing medium for Rhizobium)
  - Initialized cultures of both strains of Rhizobium using LB broth, 10% Tryptic Soy broth, and 50% Tryptic Soy broth.
- Cyanobacteria
  - Literature search to determine protein pathways that cause lagging strand pathway recombination.
- General
  - Tried to electroporate seven Biobrick plasmids into E. Coli (BBa_K125000, BBa_K274002, BBa_K274003, BBa_K274004, BBa_K274100, BBa_K274110, BBa_K274111)
  - Used protocol described in detailed Lab Notes/ Protocol section

6/11/2015

- General
  - Checked results of yesterday's electroporation. All plates showed no growth.
  - Grew up E. Coli for electroporation.
  - Tried to electroporate [1EG] into DHSalpha.
  - Made LB broth for culturing E. Coli.
- Rhizobium
  - Checkedcultures from yesterday.
  - 50% Tryptic Soy Broth showed most growth; however, 10% Tryptic Soy Broth and LB broth also showed growth.
- Cyanobacteria
  - Literature search into possible beta homologues.

6/12/2015

- General
  - Checked yesterday's E. Coli electroporation results.
  - Still no growth.
- Rhizobium
  - Started designing primers to amplify promoters (nodABCD, tauA, bacA, melA, nifH, nodF, nodA, anderson strong (constitutive))
- Cyanobacteria
  - Literature search for more beta homologues.

WEEK 3 (6/15 to 6/21, 2015)

6/15/2015

- **Cyano**
  - Frozen Stocks of *Synechococcus* strains PCC 6803 and UTEX 2973 were made
    - 500ul of 50% glycerol + 500ul of culture solution (*Synechococcus* has been growing for 1 week)
    - Freeze in -80 C

- Growing cyanobacteria cultures turned green.
  - New Masses:
    - 250 ml = 230g
    - 500 ml = 407.05g
    - 50 ml = 48.47g
    - Water was added to return cultures to their original weights as noted prior
- **Rhizo**
  - Frozen Stocks of *Rhizobium Tropici* were made
    - 500ul of 50% glycerol + 500ul of culture solution (*Rhizobium* was cultured at 30°C for 2 days (from last Wednesday) and was left at room temperature subsequently).
  - Started to check ribosome binding sites for the Rhizobia plasmids using RBS calculator
    - Maximum translation initiation rate = 10000
    - Insert the rbs before the gene and after the promoter
  - Contacted Jacobs-Wagner about R. meliloti

6/16/2015

- **Rhizo**
  - Prepared LB min in flask
    - Autoclaved LB min and pipette tips
  - Miniprep of Jaymin's plasmid pZE216
    - Note: substitute warm nuclease-free water for EB in miniprep
    - Danny did the miniprep, we observed. Final DNA concentration: 68ng/ul.
  - Electroporation of *E. coli* with Jaymin's plasmid (pZE216) and our biobrick plasmids
    - Purpose: Get all of us familiarized with electroporation, and make sure we do the transformation efficiently
    - Cultured E. coli in LB min for 3 hrs. OD = 0.611
    - Transformed using the minipreped plasmid from earlier in the day, and from biobrick plasmid solutions
  - Others
    - Recovered *R tropici* from glycerol stock
    - Cultured more cultures for miniprep tomorrow

6/17/2015

- **Cyano**

  Performed PCR to isolate Synechocystis sp. 6803 promoter isiAB
  - Purpose: PCR to isolate isiAB promoters from synechocystis PCC 6803
  - Primers used: 273 & 275 from the iGEM database on google drive
  - DNA template: full 6803 genome extracted using a Qiagen kit at 5.1 ng/uL
  - Procedure:
    - 20 uL fast HS polumerase mix was mixed with .5 uL primer 273 and .5 uL primer 275, 2 uL of PCC 6803 DNA, and 17 uL qH_2O. q

  ○ Controls were prepared:

i. nD = 20 uL polymerase mix, .5 uL both primers (1 uL total), and 19 uL qH_2O

ii. nP = 20 uL polymerase mix, 2 uL DNA template of PCC 6803, and 18 uL qH_2O

iii. Water = 20 uL polymerase mix, 20 uL qH_2O

  ○ PCR was conducted on the FAST protocol:

  5 min 95C
  15 sec 98C
  15 sec 58C
  30 sec 72C
  Repeat b-c-d 30x
  5 min 72C
  Indefinite 12C

  ○ Setup:

Label	I	I	nD	nP	W
Contents	Primers + DNA for isiAB promoter	"	No Dna, just Primers	No primers, just DNA	water

  ○ Run on gel and purified. Resulting concentration of isiAB DNA: 28.6 ng/uL.

- **Rhizo**
  - ○ Checked electroporation results from yesterday; controls have 100+ colonies but the other plasmids were not successful
  - ○ Recovered R. tropici:
    - Two liquid from -80C
    - One plate from 50% TSB room temp
    - One plate from -80C
  - ○ Obtained *S. meliloti* cultures from Jacobs-Wagner lab

1. *S. meliloti* 1021 (#356) - plated on LB, PYE, 50% Tryptic soy
2. *S. meliloti* MB501 (#370) - plated on LB, PYE, 50% Tryptic soy
   Derivative of *S. meliloti* 1021 (contains a Tn5 carrying trimethoprim resistance - http://www.researchgate.net/profile/Daniel_Gage/publication/242620268_Use_of_Green_Fluorescent_Protein_To_Visualize_the_Early_Events_of_Symbiosis_betweenRhizobium_melilotiand_Alfalfa_(Medicago_sativa)/links/0046352ceb78714254000000.pdf)
3. *S. meliloti* WM249 (#371) - plated on LB, PYE, 50% Tryptic soy

  ○ Derivative of *S. meliloti* 1021

4. *E. coli* TG1 with pMR10
5. *E. coli* TG1 with pMR20

Notes:

R. meliloti WM249 and MB501 are derivatives of Rm1021 with transposon insertions permitting the efficient electroporation of E. coli plasmid DNA. WM249 contains a Tn5-233 element encoding gentamicin resistance, whereas MB501 (obtained from M. Barnett, Stanford University) contains the sameTn5-233swapped for trimethoprim resistance.

From <http://www.researchgate.net/c/nqv8t4/javascript/lib/pdfjs/web/viewer.html?file=http%3A%2F%2Fwww.researchgate.net%2Fprofile%2FWilliam_Margolin%2Fpublication%2F14134578_Generation_of_buds_swellings_and_branches_instead_of_filaments_after_blocking_the_cell_cycle_of_Rhizobium_meliloti%2Flinks%2F02e7e52207b0b00b31000000.pdf%3FdisableCoverPage%3Dtrue>

Didn't do miniprep today.

6/18/2015

- **Cyano**
  - Diluted all new primers for isolating promoters to 100mM
  - Set up all the PCR wells for PCR tomorrow
  - PCR of promoter fragments for psaA, isiAB, nirA, and pCpG2 from Cyanobacteria PCC 6803 and PCC 7002
    - ALL: 20 uL polymerase mix, .5 uL forward primer, .5 uL reverse primer, 2 uL backbone, 17 uL qH_2O.
    - psaA: 285 and 286, PCC 7002 template
    - isiAB: 289 and 290, PCC 6803 template
    - nirA: 293 and 294, PCC 6803 template
    - pCpG2: 305 and 306, PCC 6803
- **Rhizo**
  - Checked up on plasmids and strains from Jacobs-Wagner lab
    - Sm 1021 did not grow
    - pMR20 grew, pMR10 did not
  - Recovered R. tropici strains grew
  - Did a miniprep of E. coli (pkt230 and pZE21G)
  - Checked the DNA concentration afterwards
    - pKT230 DNA concentrations: 18.8, 15
    - pZE21G concentrations: 23.3, 41, 41, 28
  - Electroporated pkt230 and pze21g into E. coli and Rhizobium
    - Only allowed recovery period of ~1hr, Rhizobium needs ~4hr recovery

6/19/2015

- **Rhizo**
  - Rhizobium electroporation did not work, but E coli worked with both pze21g and pkt230
  - Cultured tropici, 356, 370, 371 for electroporation

6/20/15

- **Cyano**
    - BACKGROUND RIFAMPICIN RESISTANCE IN CYANO

1. Determine which solvent is best for dissolving rif
    1. 50 mg of rifampicin was dissolved in 500 ul of H20, EtOH, and MeOH
    2. The antibiotic did not dissolve fully in any solvent, but was mostly dissolved in MeOH (as opposed to only marginally dissolved in EtOH and H2O)
    3. The MeOH suspension was diluted to 10 mg/ml and stored at -4 C (5 ml total)
2. Background Rifampicin Resistance Assay
    1. Conditions to test:
    2.

Strain	0 ug/ml	20 ug/ml	40 ug/ml	60 ug/ml	100 ug/ml
PCC 7002					
UTEX 2973					

All conditions will be tested in duplicate (20 plates total)

1. Adding 10 mg/ml rifampicin stock to 50 ml agar:

To Make…	20 ug/ml	40 ug/ml	60 ug/ml	100 ug/ml
…Add __ of 10 mg/ml Stock.	100 ul	200 ul	300 ul	500 ul

1. Both strains should be grown starting at the same OD

- CHARACTERIZING A BIOBRICK: T7sfGFP
- pZA21_T7sfGFP is a plasmid containing sfGFP under a promoter recognized by T7 phage RNA polymerase. It confers spectinomycin resistance to cells.
- Yesterday (6/19) a strain of ECNR2 containing the plasmid was retrieved from the -80 C freezer. The strain was grown in 5 ml LB min + spec culture overnight at 37 C.
- Today (6/20), the plasmid was miniprepped out of the ECNR2 and transformed into a strain of ECNR1 containing T7 RNA polymerase under an ATC-inducible promoter (ECNR1 B12.T7RNAP, obtained from Eddie)
    - Miniprep Results
        - Final concentration of pZA21_T7sfGFP: 53.6 ng/ul
        - Plasmid is in Qiagen EB Buffer
        - Solution of plasmid was stored in "iGEM 2015 DNA" box in -20 (iGEM Database ID: 13)
    - Transformation Results

#	Strain	DNA	Time Constant	Plate On…
1	ECNR1 B12.T7RNAP	pZA21_T7sfGFP	5.1	LB and LB Spec
2	ECNR1 B12.T7RNAP	n/a (control)	5.0	LB and LB Spec

6/21/15

- **Cyano**
  - THE BG11 PROBLEM
- PCC 7002 that was inoculated and grown in Sigma BG11 medium 48 hours ago (on Friday 6/19) shows no signs of growth yet. This could be due to the fact that the Sigma media is intended for growth of freshwater strains and PCC 7002 is a marine strain.
- A test of the 1x BG11 media indicated that it is highly acidic (pH around 5)
- On the product website, Sigma recommends adding 10 g/L of NaCl and 1 ug/L of vitamin B12 to the media to culture marine strains.
- Addressing the problem:
  - 1X BG11 was prepared and NaCl was added to a concentration of 10.0 g/mL. The solution was filter sterilized with a 0.22 um syringe-driven filter unit.
  - 200 ul of PCC 7002 stock was inoculated into about 50 mL of BG11 + NaCl media and placed in the 38 C shaking incubator with light.
  - Vitamin B12 is not available in the iGEM or the Isaacs lab (but the Groisman lab in ABC has some). If growth is unsuccessful, the Groisman lab will be contacted for some B12.

CHARACTERIZING A BIOBRICK: T7sfGFP

- Plate growth from yesterday's transformation

Plate	ECNR1 ΔtolC B12.T7RNAP + pZA21 T7sfGFP	ECNR1 ΔtolC B12.T7RNAP
LB	Lawn	Lawn
LB + Spec	~200 colonies	No growth

- 24 colonies of ECNR1 ΔtolC B12.T7RNAP + pZA21 T7sfGFP were picked from the LB + Spec plate and inoculated into 24 wells containing 150 ul LB + Spec (1x) on a 96-well plate. These colonies were allowed to grow at 32 C and 540 RPM for 3 hours.
- After 3 hours, 1 µl of culture was inoculated into 150 µl of LB
- 96-well plate setup for 12-hour growth assay:

Row	Sample	Media
A	ECNR1 ΔtolC B12.T7RNAP + pZA21 T7sfGFP	LB Spec/ATC
B	ECNR1 ΔtolC B12.T7RNAP + pZA21 T7sfGFP	LB Spec/ATC
C	ECNR1 ΔtolC B12.T7RNAP	LB Spec/ATC
D	ECNR1 ΔtolC B12.T7RNAP + pZA21 T7sfGFP	LB Spec
E	ECNR1 ΔtolC B12.T7RNAP + pZA21 T7sfGFP	LB Spec
F	ECNR1 ΔtolC B12.T7RNAP	LB Spec

- 12-hour growth assay was left to grow overnight

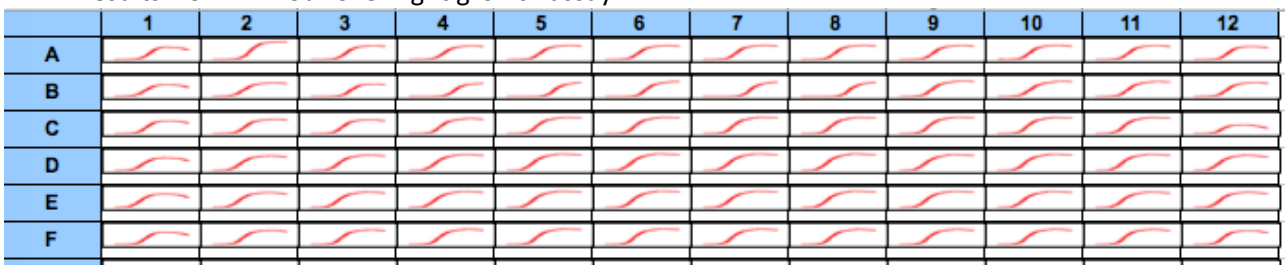
WEEK 4 (6/22 to 6/28, 2015)

MONDAY, 6/22

Meeting with faculty advisors

Characterizing a biobrick: T7sfGFP

- Results from 12-hour overnight growth assay:



- All samples exhibited similar growth
- Controls did not work:
  - Bad spectinomycin stock?
  - Cross-contamination?
  - Is ECNR1 resistant to Spec?
- To test the quality of the spec stock, ECNR1 ΔtolC B12.T7RNAP glycerol stock (iGEM Database ID 118) was inoculated into 3 ml each of LB with spec and LB without spec. DH5a interlab 3 glycerol stock (iGEM Database ID 107) was also inoculated into 3 ml LB with and without spec. Both strains should not grow in the spec culture.

RHIZOBIUM

Electroporation

- Previous electroporation with pkt230 in Rhizo worked--just takes a few days for it to grow
- Doing a new electroporation, allowing a recovery period of 4 hours
  - Time constants ranged from 4.21-4.71
- Did a second electroporation, will allow recovery time overnight
  - Time constants ranged from 4.76-4.93

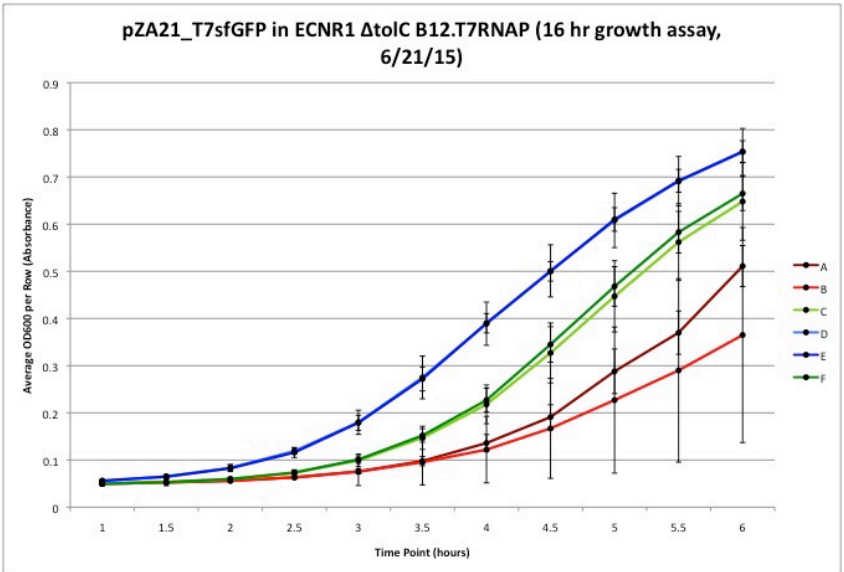
TUESDAY, 6/23

Characterizing a biobrick: T7sfGFP

- ECNR1 ΔtolC B12.T7RNAP is resistant to spec
- Results from yesterday's growth test:

Strain	LB + Spec	LB
ECNR1 ΔtolC B12.T7RNAP	Growth	Growth
DH5a (iGEM ID 107)	No Growth	Growth

- The ECNR1 ΔtolC B12.T7RNAP grown up in LB + Spec last night will be inoculated into LB + Kan and LB + Cat to test resistance.
- Found strain BL21 DE3 [T7promoter_GFP_pZE21 backbone] (iGEM ID 90) in iGEM database.
    - Glycerol stock was inoculated into 3 ml each of LB, LB/Spec, and LB/Kan (pZE21 should confer Kan resistance) and left to grow at 37 ˚C overnight
- Plan for tomorrow: Miniprep pZE21_T7GFP out of the BL21 DE3 strain and transform plasmid into ECNR1 ΔtolC B12.T7RNAP to repeat growth assay on Thursday evening
- Plated some ECNR1 ΔtolC B12.T7RNAP from refrigerator stock (made 6/20/15) to get single colonies for transformation tomorrow
- 16 hour growth assay results from pZA21_T7sfGFP in CFH 118, conducted 6/21/15:



    - Rows A and B should have had identical growth
    - Rows D and E should (and did) have identical growth
    - CFH118 with the T7RNAP induced by ATC had a slower growth rate than cell populations without the RNA polymerase induced. Cell populations carrying the plasmid without induction (D and E) actually grew faster than populations not carrying the plasmid (C and F). This could be due to the different inoculation methods used for each cell type. Will address in next growth assay on 6/25.

CYANOBACTERIA

FLP in Cyanobacteria

- Primers arrived today. Will wait until we have more PCC7002 to do the chromosome extraction and amplify the homology arms.

The BG11 Problem

- Obtained some vitamin B12 from the Crawford lab and added 1 µg/ml to the flask of PCC7002 currently growing in Sigma BG11 + 10 g/l NaCl. This makes up the media which Sigma claims will work for marine cyanobacteria strains.

Promoters

Diluted primers and prepared to PCR cyanobacteria promoters with new primers: psaA isiAB, nirA, pCpG2

Ran microprep of BbaK125000 plasmid from E. coli using QIAPrep Spin kit

RHIZOBIUM

Electroporation

- Checked electroporation #1 results from yesterday
    - Sm 356, 370, 371 grew on both plates (including negative control); could be naturally kan resistant
- Plated cultures from electroporation #2

Antibiotic Resistance Assays

- Performed the antibiotic assays for Rhizobia
- Plated 100ul of *R. tropici*, *S. meliloti* 356, 370, 371 on kan and on spec plates to look at resistance
- 96 well plate assays for resistance of *R. tropici*, *S. meliloti* 356, 370, 371 to Kan, Carb, Cam, Spec
    - Diluted antibiotic 50x, 100x, 200x, 400x, 800x, 1600x (we usually use 1000x)

WEDNESDAY, 6/24

- met with Professor Dellaporta about high-throughput ligation-independent cloning (LIC) method

Characterizing a biobrick: T7sfGFP

- Plasmid pZE21_T7GFP was miniprepped out of BL21 DE3 (125 µl total at a concentration of 57.0 ng/µl in buffer EB)
- Transformation of pZE21_T7GFP into CFH 118 did not work (electroporation machine problems), so will retry tomorrow

CYANOBACTERIA

Promoters

- PCR amplified cyanobacteria promoters (psaA isiAB, nirA, pCpG2) with FAST cycle (give protocol) — no gel electrophoresis because power went out

Electroporation

- Electroporation of K125000 into E. Coli strain DH5Alpha — low time constant of 3.85

RHIZOBIUM

Antibiotic Resistance Assays

- None of the strains grew on kan plates
- *R. tropici* and *S. meliloti* 371 grew on spec plates
- 96 well plate assays: Not enough growth yet. To see tomorrow

Electroporation

- Checked 2nd electroporation results; no new growth
- Did another EP (2) with k125000

Rifampicin assay: to see if we could use MAGE to induce resistance to rifampicin

- Plated *S. meliloti* 356, 370, 371 on Tryptic Soy + rifampicin plates (20ug/ml, 40ug/ml, 60ug/ml, 100ug/ml)
- Plated *S. meliloti 356, 370, 371* on Tryptic Soy plates at 2*10^7 and 2*10^8 dilutions

FLP Knockout in Rhizobium

- Finalized primer list for building the FLP KO construct; primers will get ordered by the end of the week.

THURSDAY, 6/25

Characterizing a biobrick: T7sfGFP

- Plasmid pZE21_T7GFP was transformed into ECNR1 ΔtolC B12.T7RNAP, following the electroporation protocol outlined in the "Protocols" tab:

#	Strain	OD600	DNA	TC	Plate On
1	ECNR1 ΔtolC B12.T7RNAP	0.492	pZE21_T7GFP	4.8	LB/LB+Kan
2	ECNR1 ΔtolC B12.T7RNAP	0.492	n/a (control)	5.1	LB/LB+Kan

- Allowed cells to recover for 75 min in LB media at 37 ˚C
- Also plated ECNR1 ΔtolC B12.T7RNAP that was used to grow samples up to mid-log phase, to have during growth/expression assay tomorrow evening.

CYANOBACTERIA

Promoters

- Gel electrophoresis of cyanobacteria promoters (psaA isiAB, nirA, pCpG2) showed smudges in all wells and the promoter was most likely not amplified correctly.
- Power went out during PCR, so it had to be run twice, and too much primer (0.5 uL of 100 mM primer) was used in the mix. Need to redo experiment.

RHIZOBIUM

Electroporation results:

- CIAT and sm 371 have spec resistance

Antibiotic plate assay:

- OD not high enough to measure yet
- Same results as the day before
- None of the strains grew on kan plates
- *R. tropici* and *S. meliloti* 371 grew on spec plates
- 96 well plate assays: To check again tomorrow

PCR Amplification of Rhizobium Primers:

- Purpose: PCR to isolate nodABCD, bacA, melA, nifH, nodF, nodA promoters from *S. meliloti* 1021
- DNA template: *S. meliloti* #356 from plate
- Mix: 25ul Kapa HiFi mix, 22ul nuclease free water, 1ul Forward primer, 1ul Reverse primer, template
- Primers: 329 and 330 for nodABCD (wells 1-2), 335 and 336 for bacA (wells 3-4), 338 and 339 for melA (wells 5-6), 341 and 342 for nifH (wells 7-8), 344 and 345 for nodF (wells 9-10), 347 and 348 for nodA (wells 11-12)
- PCR was conducted on the FAST protocol:
  a. 5 min 95C
  b. 15 sec 98C
  c. 15 sec 55C
  d. 30 sec 72C
  e. Repeat b-c-d 30x
  f. 5 min 72C
  g. Indefinite 12C
- To run gel and extract DNA tomorrow

Amplification and Gibson Assembly of plasmids to remove EcoRI site from sfGFP

- sfGFP from pUC57-Kan (2.7kb plasmid) and sfGFP (with his Tag) in T7-sfGFP construct in pZA21 have EcoRI site in them.
- Goal: Amplify with primers that remove the site while keeping amino acid sequence constant. Then do Gibson Assembly to recircularize plasmid
- Mix: 25ul Kapa HiFi mix, 22ul nuclease free water, 1ul Forward primer, 1ul Reverse primer, 1ul template
  - 2 tubes with 1ul pUC57-Kan (tubes 1 and 2), 2 tubes with 1ul pZA21 (tubes 3 and 4)
  - Primers: 325, 326
- PCR was conducted:
  - 5 min 95C
  - 15 sec 98C
  - 15 sec 55C

- 180 sec 72C
- Repeat b-c-d 30x
- 5 min 72C
- Indefinite 12C

- Digest for 1 hour with DPN1 (1ul of enzyme per tube) at 37C, before heating for 20 min to denature enzyme.
- Added 7ul of loading dye, ran 1% gel, 150V for 40 mins.
- Unfortunately, bands were not clear. Decided not to go ahead with gel extraction and Gibson Assembly.
  - Solution: 2 step PCR - amplify at 55C for 6 rounds, then do remaining rounds at 67C (since we have the overhangs for Gibson assembly which can bring Tm to 72). In the future, should design primers so they anneal at higher temperate (say 60C)
  - Also: Dilute plasmid to something less than 1ng/ul (then use 1ul of this)
  - Note: Primers used were at 100uM (started PCR before we realized we should be working with 20uM)

<u>16S rRNA sequencing</u>

- Goal: to verify species identity for R. tropicii and S. meliloti
- Template: Amplified rhizobia plated from glycerol stock (tube 1 to 4), rhizobia that we are currently using (from June 23 - tubes 5 to 8), *R. tropici* that grew on spec plate (for antibiotic assay), *S. meliloti* 356 that grew on kan plates as a lawn
  - From stock: Tube 1: *R. tropici*, 2: *S. meliloti 356,* 3: *S. meliloti 370,* 2: *S. meliloti 371*
  - From 6/23 culture: Tube 5: *R. tropici*, 6: *S. meliloti 356,* 8: *S. meliloti 370,* 8: *S. meliloti 371*
  - Tube 9: *R. tropici* that grew on spec plate (for antibiotic assay), 10: *S. meliloti* 356 that grew on kan plates as a lawn
  - Either colonies picked from plate, or liquid culture diluted 20x in nuclease free water
- Primers:
  - 16s: 940 and 941
- Program: METHOD CALC || HOTLID 105, 30 || VOLUME 20 || TEMP 95.0, 180 || TEMP 95.0, 15 || TEMP 57, 15 || TEMP 72.0, 60 || GOTO: 2, 26 || TEMP 72.0, 300 || TEMP 12.0, 0 || END
- 20uL rxns
- Used K2G Fast
- In each tube: 10ul KAPA Fast mix, 8ul nuclease free water, 0.5ul Forward primer, 0.5 Reverse Primer, Template
  - Ran 4ul of the PCR mix in a gel. PCR from colonies gave much stronger bands
  - Sent the remainder for sequencing after diluting 2x (so we have both forward and reverse sequencing)
  - Note: Primers used were 100uM (before we diluted to 20uM)

<u>FLP Knockout in Rhizobium</u>

- Obtained about 4 ml each of R. tropici, SM1021.356 (noncompetent), SM1021.370 (electrocompetent), and SM1021.371 (electrocompetent) from Lionel.
- Used 1.5 ml of each of these cultures to perform a total DNA extraction (Qiagen DNeasy purification kit). The SM1021 strains all had similar growth (well past mid log phase), but the R. tropici was less optically dense.
  - Allowed cells to lyse in Qiagen Buffer ATL at 56 ˚C for about 2.5 hours

- Final concentrations (in Buffer AE, 200 µl of each sample):

Organism	DNA Concentration (ng/µl)
CAIT (*R. tropici*)	17.8
SM1021.356	249.7
SM1021.370	36.8
SM1021.371	43.4

FRIDAY, 6/26

CHARACTERIZING A BIOBRICK: T7sfGFP

- Results from yesterday's transformation of pZE21_T7GFP into ECNR1 ΔtolC B12.T7RNAP:

Sample	LB	LB + Kan
ECNR1 ΔtolC B12.T7RNAP + pZE21_T7GFP	Lawn	~30 colonies
ECNR1 ΔtolC B12.T7RNAP (control)	Lawn	No growth

- Growth Assay:
  - 24 colonies were picked from each of ECNR1 ΔtolC B12.T7RNAP + pZE21_T7GFP and ECNR1 ΔtolC B12.T7RNAP plates and inoculated into 150 µl of LB + Kan or LB + Spec, respectively. The colonies were allowed to grow on a 96 well plate for 4 hours (540 rpm, 32 ˚C).
  - Plate for 16-hour growth assay:

Row	Strain	Plasmid	Media	Antibiotic	Inducer
A	ECNR1 ΔtolC B12.T7RNAP	pZE21_T7GFP	LB	Kan	ATC
B	ECNR1 ΔtolC B12.T7RNAP		LB	Kan	ATC
C	ECNR1 ΔtolC B12.T7RNAP	pZE21_T7GFP	LB	Kan	
D	ECNR1 ΔtolC B12.T7RNAP		LB	Kan	
E	ECNR1 ΔtolC B12.T7RNAP		LB		

  - Plate was placed on BioTek plate reader
    - Program on Gen5: "OD600_Kinetic" (not "OD600_Kinetic_mira")
    - 16 hour run, OD read every 10 minutes
    - Set temperature of 34 ˚C

CYANOBACTERIA

The BG11 Problem

- ATCC 1047 Medium was prepared according to the recipe
- Autoclaving things:
  - Straight seawater does not seem to precipitate when autoclaved.
  - 50% Sigma BG11/50% seawater turns milky when autoclaved.
  - 100% Sigma BG11 seems to form precipitates a few days after being autoclaved.

- **Best method for preparing ATCC 1047:**
  - Autoclave some seawater and allow it to cool
  - Prepare a mixture of 75% autoclaved seawater and 25% DI H_2O
  - Add the ATCC medium chemicals directly to the seawater/DI H_2O solution, either on a stirring plate or by mixing *thoroughly* after the addition of each chemical
  - Add in Trace Metals A-5 to 1x concentration
  - Add in 20 µg/l of vitamin B12
  - Filter-sterilize the entire solution with a 0.22 µm filter
- **Best method for preparing 50% seawater/50% BG11:**
  - Autoclave some seawater and allow it to cool
  - Mix seawater with previously prepared 1x Sigma BG11 in equal proportions to obtain a 50/50 solution
  - Add in 20 µg/l of vitamin B12
  - Filter-sterilize the entire solution with a 0.22 µm filter
- **Best method for preparing 1x Sigma BG11:**
  - Mix 50x BG11 stock with previously autoclaved MilliQ water to a final 1x concentration
  - Filter-sterilize the entire solution with a 0.22 µm filter

Promoters

- Re-ran PCR amplification of cyanobacteria promoters plus E. coli promoters (pBAD, LambdaCI) with primers diluted to 20 mM.

RHIZOBIUM

Electroporation

- Rhizobium electroporation results
  - Kan plates: no growth
  - Spec: 370, kan have resistance

Rifampicin Assay for *S. meliloti (356, 370, 371)*

- Worked with 2*10^10 cells
- Below limit of detection even with 20ug/ml Rifampicin
- **To try at lower concentrations**
  - Want to find optimum where resistant cells grow, but not susceptible ones
  - Might use UV to induce mutagenesis

16S rRNA sequencing

- Confirmed that S. meliloti 356, 370, and 371 stock are S. meliloti 1021 and R. tropici stock is R. tropici.

mutS Knockout in Rhizobium

- Revised primers to have enough homology between amplified sequences for Gibson assembly

Conjugation

- Goal: Transform pKT230 from DH5-α *to S. meliloti* 371.
- Refer to http://www.nature.com/nprot/journal/v9/n10/full/nprot.2014.081.html, Steps 65 to 86.
- Summary:
- 1. Grow confluent culture of DH5-α with pKT230 and confluent culture of *S. meliloti* 371.
- 2. Wash with 1ml LB.
- 3. Repeat wash. Resuspend in 100ul LB.

- 4. Mix 50ul DH5-α with 50ul of *S. meliloti* in microtube. Spot 20ul x 2, 10ul x 6 (8 spots in total) on LB plate. Incubate at 30C for 1hr.
- 5. Wash off the spots from the plate with LB, and plate on TSB + Kan + Spec (select for plasmid and for rhizobium).
- 6. Also streak controls: DH5-α, *S. meliloti*.

WEEK 5 (6/29 to 7/5, 2015)

Colin - 6/29/15

CHARACTERIZING A BIOBRICK: T7sfGFP

- Conducted a DPN1 digest on Lionel's amplified T7sfGFP's without the EcoR1 restriction site. There was not enough DPN1 to digest each sample in duplicate, so the samples are ordered on the gel as follows:

Nat + DPN1	Nat	pZA21 + DPN1	pZA21	pZE21 + DPN1	pZE21	-ze + DPN1

- Gel did not work… Very faint bands around 3 kb (where the plasmid is expected), but not enough to extract and isolate.

GEL POURING TIPS

- Use large well comb for cloning experiments
- Sybr-Syc is concentrated at 10,000x and the gel should appear slightly reddish when it is at 1x concentration
- EtBr is concentrated at 100,000x and the gel should NOT appear reddish at 1x concentration
- The small cloning gel molds take 150 ml of agarose. Use 1 g of agarose powder per 100 ml of TBE to make gel

RIFAMPICIN ASSAY IN RHIZOBIUM

- New plate concentrations to test:

Concentration	Volume of 10 mg/ml stock needed to make 50 ml (µl)
0.5 µg/ml	2.5
1 µg/ml	5
2 µg/ml	10
5 µg/ml	25
10 µg/ml	50

THE BG11 PROBLEM

- PCC 7002 Cultures prepared to date:

Date	Medium	Volume of Media	Volume of Inoculant
6/26	ATCC 1047	50 ml	500 µl
6/29	ATCC 1047	50 ml	1000 µl
6/29	ATCC 1047 pH Adjusted (8.5)	50 ml	500 µl

- **TO ADJUST pH OF ATCC 1047 MEDIA**: Add 6 µl of 1 M KOH solution per 10 ml of media being prepared (i.e. 30 µl of 1 M KOH if preparing 50 ml of ATCC 1047 media). The final pH should be 8.5.

Dan - 6/29/2015

Running PCR and gel electrophoresis

Promoter	psaA	isiAB	nirA	pCpG2	pBAD	Lambda CI
Expected Length (bp)	250	206	170	237	1364	2646
Concentration after purification	10.0 ng/uL	13.2 ng/uL	N/A	10.1 ng/uL	21.1 ng/uL	N/A

1% gel was made with
200 mL TBE
2g Agarose
20 uL sybersafe

ALL PROMOTERS ARE IN THE "CYANO" BOX IN THE -20

July 29th
TO-DO:
PCR Amplify NirA and CpcAB
USE GRADIENT PCR
Take pictures for growth on plates, both transformation and CO2 test (Remember that Control is compromised, if growth, may not be algae. Only whole liquid wash, not 50 micro)

Successfully used a gradient from 53 to 63 to amplify NirA and CpcAB
Gel extracted the PCR product using the Qiagen kit.
Gained yields of ~30 ng/ul
Ran a Gibson reaction

Took photos of Cyano growing

Holly – 6/29/2015

- Made a 1% sybr- safe gel
- Goal: to make a gel for PCR cloning
- Procedure:
  a. Use the small gell tray and the comb with broad wells
  b. Use microwave to heat up 80 uL of TBE with 0.8g of agarose for 1.5 min
  c. Let cool for 5 mins (until no fumes)
  d. Add 8 uL of sybr- safe gel
  e. Pour into gel tray; comb down the bubbles
  f. Let sit for 30-40 min until cool

- Conjugation:
  - Tried conjugating Sm 371 with pKT230 (DH5a)
    - 371 is resistant to kan, so this did not work
  - For k125: need a helper plasmid RP4
    - Triparental mating
    - ATCC strain with helper plasmid, will try conjugation with k125

ELIC:

- Purpose: to transfer in gene-of-interest (beta homologue) into plasmid
  - Back up method for Gibson
- Notes:
  - Plasmid should have 2 restriction sites that cannot anneal to each other
  - 4:1 ratio of plasmid: insert
  - 60 min recovery
  - sfGFP has T7 promoter; will test this with ELIC
  - Using pZE21G as the plasmid, has EcoR1 cut sites
    - Put cut sites in primer overhang
  - Promoter is outside of the restriction site, so only need to restore rbs
  - Treat with cip to remove the 5' phosphate

DH5a electroporation:

- Purpose: EP w/ pZE21G for miniprepping for ELIC; EP w/ k125000 to see if the plasmid works
- BTX electroporator:
-

Cuvette	Time constant
K125	4.53
Pze21G	4.53
-ve	5.01

- Resistances:
  - Pze21g: kan
  - K125000: spec

Growth curve assay:

- Purpose: figure out growth curve of Rhizo strains so know when mid-log is for EP
- Procedure

Use a 96-well plate. Each row should have 100 uL TSB

Row	Strain
A	CIAT
B	Sm 356
C	Sm 370
D	Sm 371
E	-ve

Take colonies from stock plates, put into appropriate well
Incubate
Transfer 1 uL from each well into a new plate containing 150 in each well
Observation: CIAT did not grow yet. Other meliloti strains looks cloudy.
Plate reader (BioTek Gen 5) settings:
Kinetic, 30C, 40 hours, read at 10 min intervals
Shake: orbital 425 cpm, continuous, read absorbance, OD 600
Check results on Friday 8:30 AM

Lionel - June 29

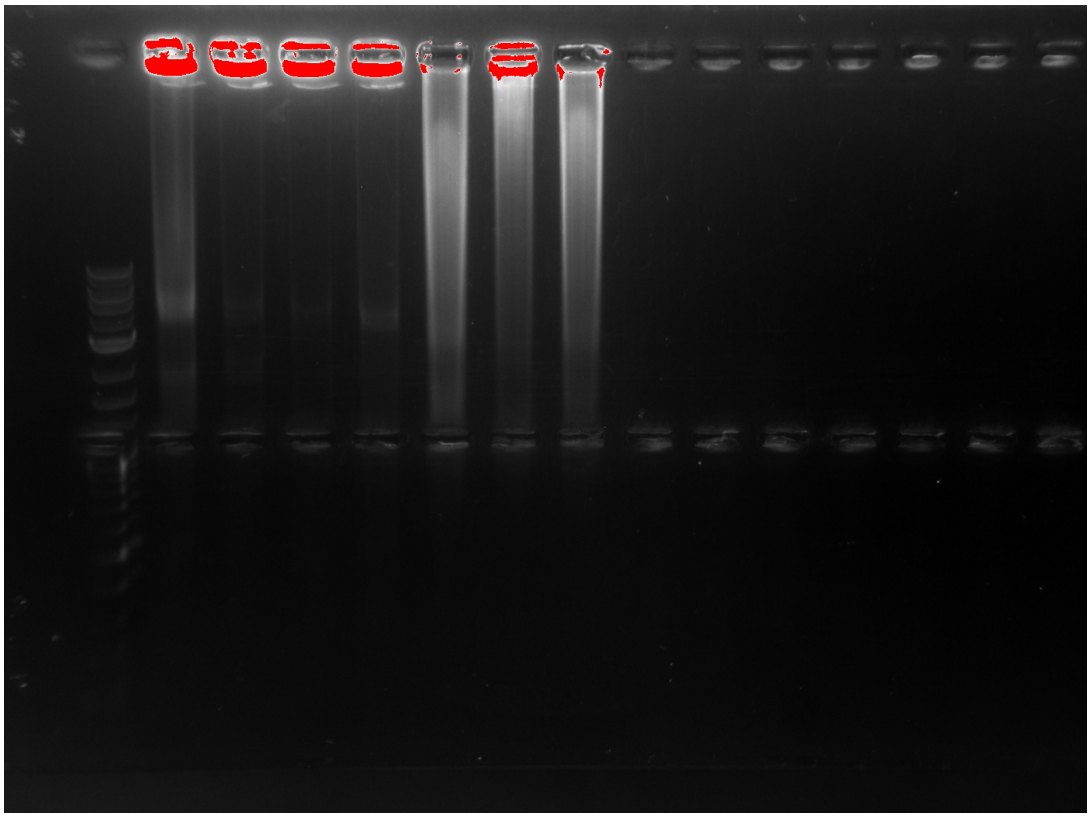
Results from previous days:

Conjugation

- Conjugated plate grew, but so did *S. meliloti* on Kan + Spec (resistant to both)
- Could try *with R. tropici* since it is is resistant to spec but not to kan. Any other method to select for rhizobium?
    - Jessica: M9 Sucrose plates!

PCR to edit GFP EcoRI site

Two step PCR



Still not efficient

Dan - July 30th

TO-DO:
Amplify gibson assembled linear fragments to see if successful
Gibson Assemble Dan's promoters
Check transformation growth

Colin - 6/30/15

MAGE PROTOCOL
(Pulled form Ariel's protocol; also obtained the Nature Protocols paper (2014) describing the MAGE procedure in detail)

1. Start 2, 3ml cultures in LB min plus carb, per strain, and grow at 34C shaking to mid log (0.4-0.5 OD) (~4 hours).
    At 37C, l red is overactive and is toxic to cells.

2. While cells growing:
    Make and chill 50 λ solutions at 1µM each (1 λ 50uM stock, into 49λ DI H2O) (0.5-1µM is optimal).
    Chill electroporation cuvettes

3. Before de-repressing λ red, freeze down 1ml for a backup stock (1ml cells + 200 l 80% glycerol, and store at -80C). Transfer remaining 2 ml of cells to shaking 42C water bath: 250 rpm, 15 mins, which de-represses λ red genes.

4. Spin down 1 ml of this culture in the cold room at 2/3 max (10-11) for 30 seconds. Wash pellet in DI water 2x, and then re-suspend pellet in 1 µM oligo solution.
    Leave remaining 1 ml of culture in the cold room in case something happens

5. Electroporate bugs plus oligos in cuvette at 2.5kV, 100 ohms, 25 uF, and record time constant. Add immediately to 3ml LB and recover culture for at least 2 replication cycles at 30C (until they reach mid log, or ~2 hours). After the 2 hour recovery make a frozen stock.

6. From the remaining two ml, make serial dilutions and plate.

**When starting the additional cycle immediately after the last one, after the 2 hour recovery period, make a frozen stock as stated before, and begin cycle again by derepressing l red.
**If you want to start a new cycle in the morning, after the 2 hour incubation period, leave the culture growing overnight, and in the morning, back dilute 30 l into 3 ml, and wait until the culture reaches mid log.
**If you want to start a new cycle after the weekend, after the 2 hour incubation period, leave the culture in the cold room.

LIONEL'S DNA

- Did a gel extraction of some of Lionel's DNA samples
  - Final concentrations:

Sample	Concentration (ng/µl)
1	20.4
2	293.9
3	81.2
4	19.6
5	25.7
6	8.5
7	20.5

QUANTIFYING CYANOBACTERIA GROWTH

- Difference between OD 730 and 750?
  - Measuring the same samples of bacteria at different OD's
    - The UTEX strain measured looked significantly greener and slightly darker than the sample of PCC 6803 tested

Strain	OD 600	OD 650	OD 730	OD 750
UTEX 2973	2.100	1.885	1.326	1.290
PCC 6803	0.866	0.880	0.796	0.742

  - There doesn't appear to be a significant difference between OD 730 and 750; OD 730 readings seems to be slightly higher.
  - OD 730 might be slightly more sensitive and is used in the UC Chile and Peccia lab transformation protocols, so let's go with that from now on.
  - Reading at OD 600 definitely confounds with chlorophyll concentration readings, which is typically done at 650nm.
  - As expected by qualitative observations of sample greenness, the UTEX strain has a much higher chlorophyll concentration than PCC 6803.

PREPARING FOR CYANOBACTERIA TRANSFORMATIONS

- 15 ml cultures of UTEX and 6803 were inoculated to grow overnight

Strain	Starting OD 730	Ending OD 730 (after ON growth)
UTEX	0.408	0.503
6803	0.378	0.505

Erin - Tues. 6/30/15

ELIC To-Do:

1. Electroporate DH5alpha with pZE21G. Grow up 5 mL culture to confluence overnight.
2. Miniprep pZE21G in one tube.
3. Design and order 2 primers for ELIC.
4. Restriction digest EcoRI sites on pZE21G.
5. ELIC - cotransform pZE21G and amilCP insert into E. coli (strain:)

Started 1 mL culture of DH5alpha at 11:00.

Electroporation at 13:30.

	Plasmid	Resistance	Results	Expected?
1	pZE21G	Kan	Did not plate	
2	K125000	Spec	No colonies	no
3	Neg. control	none	No colonies	yes

Notes:

- E. coli used to transform 2 and 3 were not fresh cultures. They had been sitting on the bench at RT for several hours.
- Plasmid concentration may be very low.

Made F and R primers for ELIC pZE21G and amilCP.

Lionel - June 30

- Diluted in nuclease free water new primers that we received (100uM stock and 20uM working solution)

<u>PCR of Promoters</u>

3 tubes each: 356, 370, 371
nodABCD (primers 329 and 330): tubes 1 to 3
melA (+ve control) (primers 338 and 339): tubes 4 to 6
nifH (primers 341 and 342): tubes 7 to 9
nodA (primers 347 and 348): tubes 10 to 12

<u>PCR of GFP with Gibson Overhangs</u>

nodABCD (primers 328 and 331): tubes 13 and 14
bacA (primers 328 and 337): tubes 15 and 16
melA (primers 328 and 340): tubes 17 and 18
nifH (primers 328 and 343): tubes 19 and 20
nodF (primers 328 and 346): tubes 21 and 22
nodA (primers 328 and 349): tubes 23 and 24

Note: primer 328 is the GFP sequencing forward primer (no overhangs)

Danny - July 31st

Importance of CO2

After three days, lawns have grown on the plate placed in the CO2 incubator. Only small early colonies have formed on the plate placed in the shaking ncubator.

CO2 IS VERY IMPORTANT FOR GROWTH.

Colin - 7/1/15

CYANOBACTERIA TRANSFORMATION

- Followed a mix of the UC Chile and Peccia lab protocols. Did this procedure for both UTEX and 6803.
- 14 ml of ON culture (OD's above) were spun down (2750 x g, 4 ˚C) in 15 ml falcon tubes for 10 mins
- Cells were washed
    - Pellet was resuspended in 300 µl cold fresh BG11
    - 2700 µl BG11 was added and mixed with resuspended culture
    - Cells were again spun down at 2750 x g, 4 ˚C, 10 mins
- Pellet was resuspended in 500 µl cold BG11
- 150 µl of cells was aliquotted into 3 1.5 ml eppi tubes
- Plasmid DNA was added to a final concentration of 2 µg/ml
    - Samples were vortexed lightly
- Samples were placed inside 38 ˚C incubator for 6 hrs, and were taken out to be vortexed lightly halfway through
- Samples:

	1 (control, no DNA)	2 (pKT230)	3 (pZE21G)
UTEX			
6803			

    - pZE21G is Jaymin's plasmid, which has a ColE1 ORI that probably won't work in cyano, but it's worth a try
- UC Chile protocol requires tons of DNA (30 µg/ml), so more pKT230 will be grown and prepped to try this protocol again with a higher DNA concentration

RHIZOBIUM GROWTH ASSAY

- ON culture from yesterday was inoculated into a fresh 96-well plate for a 40-hour kinetic OD600 assay
- Plate configuration:

Row	Strain
A	CAIT
B	SM 1021.356
C	SM 1021.370
D	SM 1021.371
E	None (control)

    - 1 µl of ON culture was inoculated into 150 µl of tryptic soy broth in each well

Erin - Wed. 7/1/15

Miniprepped 5 mL DH5alpha culture containing pZE21G (electroporated yesterday) -> 24.4 ng/uL concentration

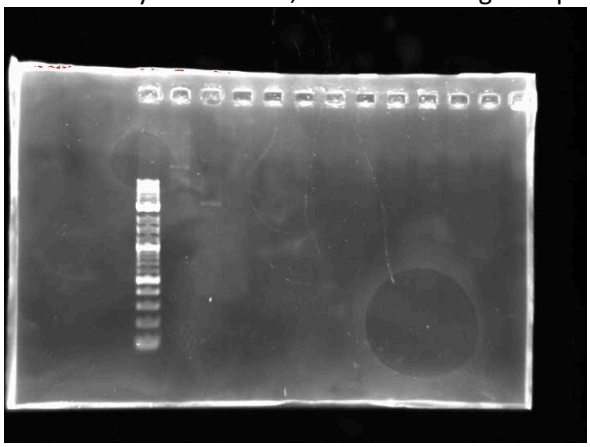
Started three 1 mL DHSalpha cultures to redo electroporation of K125 at 10:30.

Electroporation not done.

Notes:

- K125 is not transforming.
    - Miniprep did not work -> try maxiprep with 500 mL culture with K125
    - Run gel of K125 plasmid

Ran gel of K125000 and pZE21G (miniprepped 7/1 aka earlier today)
Ladder (3 uL) | K125000 (2 uL + 1 uL loading dye) | pZE21G (2 uL + 1 uL loading dye)
Results: very faint bands, so we are doing maxiprep



Made 500 mL terrific broth for maxiprep (autoclave 15 min at 121 deg C)
Inoculated 1 mL liquid culture of e. coli w/ K125000 to grow overnight

Holly - 01Jul2015

- Purification Gel
  - Goal: figure out if miniprep of the plasmid was successful
    - Plasmids: pZE21G, k125000
  - Procedure:
    - Use the thin gel combs and the small cast
    - Mix 80 mL TBE, 0.8g agarose in an erlenmeyer flask
    - Microwave for 48 sec, cool until there are no more fumes (test this by placing the opening of the flask on the microwave door)
    - Add 8 uL of sybr safe to the flask
    - Load 2 uL DNA (plasmid) + 1 uL of loading dye into PCR tubes
    - Transfer contents of PCR tubes into the gel. Add ladder (same volume as DNA)
    - Run the gel at 150 V for 20 min
- Midi-Prep
  - Goal: because k125 has a low copy number, do a midi-prep to extract more of the plasmid
  - Before doing midi-prep, need to grow up 500 mL of E. coli
  - Culturing Protocol:
    - Add 500 mL of milliQ water to large flask
    - Add 23.7g Terrific Broth
    - Add 2 mL glycerol
    - Autoclave the solution at 121 C for 15 min
    - Pour in 1 mL of E. coli k125 culture (once solution has cooled)
    - Add 500 uL spec to select for the plasmid

Jessica - 01JUL15

Results: electroporation of DH5a with K125

- No growth
- Possibly bad culture? Possibly low copy plasmid not working?

Ran PCR of K125

Mini Prep of pZE21G from 5mL DH5a

Mini Prep of K125 from 5mL DH5a

Terrific Broth for Maxi Prep

Colin - 7/2/15

CYANOBACTERIA

- PCC 7002 is growing!!!
  - Slightly greenish cloudiness is apparent in the non-pH-balanced ATCC 1047 sample (1000 µl inoculation)
  - Took about 5 days to reach this stage
- New Transformations
  - Miniprepped overnight culture of pKT230 grown in E. coli
  - Erin obtained pETcoco (10 ng/µl), which should transform into cyano

CYANOBACTERIA TRANSFORMATION

- 100 µl of each of yesterday's samples were plated onto Sigma BG11 slant agar tubes. These should dry out a lot more slowly than plates.
- The slants were placed in the 38 ˚C shaking incubator with light.

RHIZOBIUM GROWTH ASSAY

- A problem occurred with the kinetic read assay… The plate seemed to have jumped out of the holder, messing up the reads and halting the program 3 hours in.
- Assay will be re-attempted over the weekend. New ON culture is needed.
- Plates for continuing the rifampicin resistance assay will be prepared once Lionel has grown up more rhizo

TRANSFORMING pETcoco INTO E. COLI FOR AMPLIFICATION

- Also transformed pUC57-Kan_sfGFP (NJM, iGEM Database ID 15) to amplify… Stock was at a very low concentration (1 ng/µl).
- 57 µl final volume for each sample
- Samples were recovered in 1 ml LB and were allowed to grow at 37 ˚C for 1 hour before plating

Sample	Strain	DNA (Concentration µg/ml)	Resistance	Vol. DNA Used (Final Concentration ng/µl)	TC	Plate On
1	DH5α	n/a (control)	n/a	n/a	5.1	LB, LB/Carb, LB/Kan
2	DH5α	pETcoco-2	Amp	5.7 µl (1 ng/µl)	5.2	LB, LB/Carb
3	DH5α	pUC57-Kan_sfGFP	Kan	10.4 µl (0.2ng/µl)	5.2	LB, LB/Kan

Dan - 7/2/2015

Preparing Citrine PCR
Got the Citrine plasmid from Andrew in Stephen Dellaporta's lab. PYU 3034 is in the -20 in the Cyano Box.
Need to isolate citrine for Gibson assembly with 7 promoters we have.

PCR mix ALL PCR TUBES:

-20 uL FAST HS/KAPPA polymerase mix
-.5 uL forward primer 20 uM noted in the setup
-.5 uL reverse primer 20 uM noted in the setup
-2 uL backbone (assume low concentration i.e. <10 ng/uL)
-17 uL qH_2O
Total: 40 uL

Experiment 1: using primers to amplify citrine
Setup:

(overhang) Citrine	(psaA) Citrine	(isiAB) Citrine	(pCpG2) Citrine	(pBAD) Citrine	(Andsn St) Citrine	(Andsn md) Citrine	(Andsn wk) Citrine
Primers used:	283, 287	283, 291	283, 307	283, 299	283, 351	283, 352	283, 353
Concentration after purification	4.2				N/A	N/A	

Control: just primers, no backbone i.e. 7 control tubes as well.

Experiment 2: using promoters with citrine overhangs to amplify citrine
Setup:

Promoter	psaA and Cit	isiAB and Cit	pCpG2 and Cit	pBAD and CIT
Primers used	283, psaA promoter	283, isiAB promoter	283, pCpG2 promoter	283, pBAD promoter
Concentration after purification				

Control: just primers, no backbone i.e. 4 control tubes as well.

Experiment 1 PCR setup:
Volume: 40 uL

1. 5 min 95C
2. 15 sec 98C
3. 15 sec 58C
4. 20 sec 72C
5. Repeat b-c-d 6x
6. 15 sec 98C
7. 15 sec 65C
8. 20 sec 72C
9. Repeat f-g-h 24x
10. 5 min 72C
11. Indefinite 12C

Experiment 2 PCR setup:
Volume: 40 uL

1. 5 min 95C
2. 15 sec 98C
3. 15 sec 58C
4. 30 sec 72C
5. Repeat b-c-d 6x
6. 15 sec 98C
7. 15 sec 65C
8. 30 sec 72C
9. Repeat f-g-h 24x
10. 5 min 72C
11. Indefinite 12C

Holly - 02Jul2015

- Hi-Speed Midi Prep of k125000
  - Goal: extract k125000 from *E. coli*
  - Procedure:
    - Followed Qiagen kit
    - Last step: elute into eppendorf tube
    - Separate contents into two tubes, 500 uL/tube
    - Place tubes in dry ice for ~3 min to concentrate the DNA
    - Cover the top of the tubes with kim wipes (tape them down) so that DNA will not evaporate in the vacuum
    - Place in lyophilizer for 1-2 hours
      - Check at 3:30-4:30 PM
  - DNA concentration = 1.94
- ELIC

Colin - 7/3/15

TRANSFORMATION RESULTS

Condition	LB	LB/Kan	LB/Carb
DH5α	lawn	no growth	no growth
DH5α + pETcoco-2	lawn	n/a	no growth
DH5α + pUC57-Kan_sfGFP	lawn	1000 +	n/a

- Transformation of pETcoco-2 didn't work… Could we have gotten pETcoco-1 instead? Does it take a really long time to grow up? Plate was left in the 37 ˚C incubator in case this is true.
- Can retry a transformation with the teeny tiny bit of plasmid left in stock on Sunday.
- Can also try streaking some DH5a + pETcoco on LB onto an LB/Cat plate. If this grows, than plasmid we have is pETcoco-1 (which is unlikely).

HELPER PLASMID

- Inoculated single colonies containing helper plasmid (from plate "Helper Plasmid 1341") into 3 x 4ml for miniprep and making frozen stock

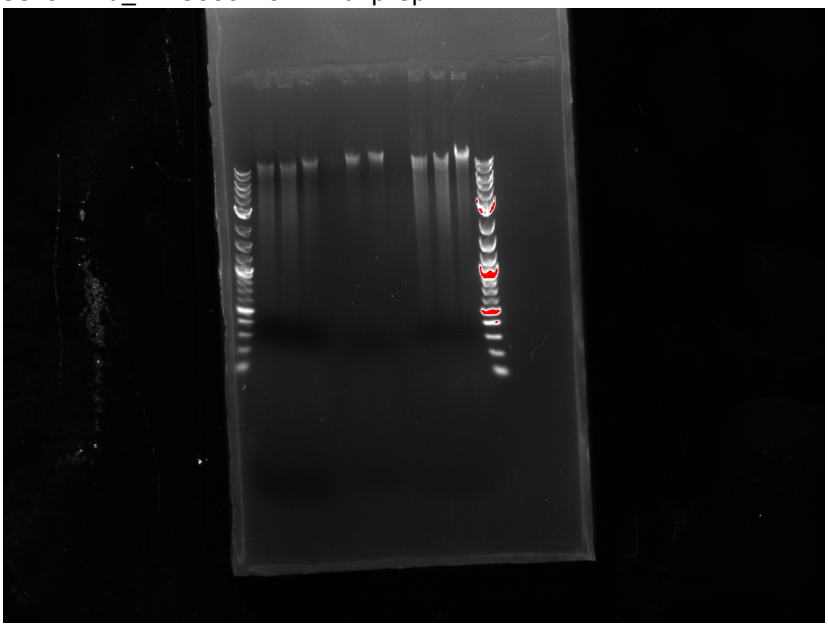
RHIZOBIUM GROWTH ASSAY

- Did not start a second growth assay trial due to plate reader technical problems… Reader reports that "absorbance signal is out of range."

WEEK 6 (7/6 to 7/12, 2015)

Monday 7/6

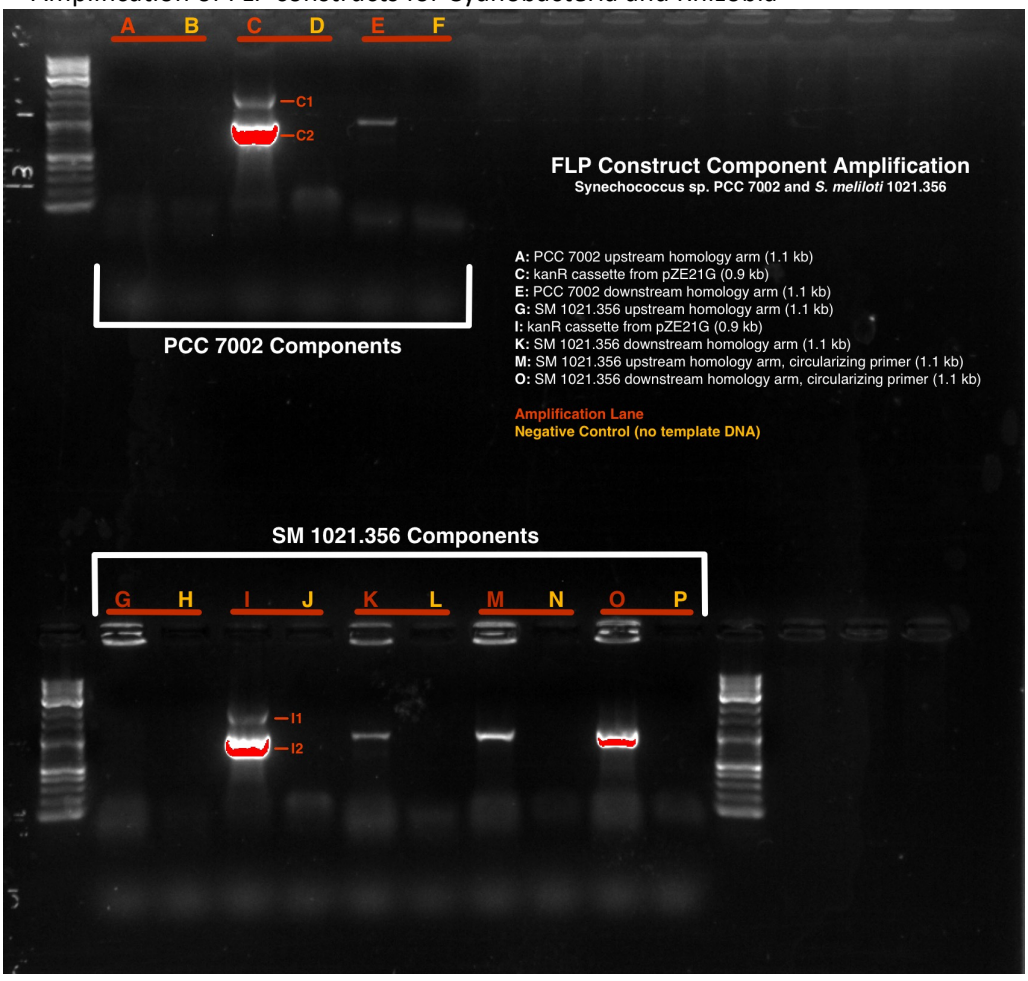
- Meeting with PIs
  - Considering ELIC and LIC as methods of inserting DNA constructs into pKT230
  - Troubleshooting cyanobacteria media recipe
- Begin testing the ELIC protocol in E. Coli MG1655 using expression of amilCP (blue chromoprotein) in K125000
  - Received MG1655 and MacH1_amilCP plates from Ariel for ELIC
  - Restriction digest K125000 at EcoRI (one site) BsaAI (two sites) sites
    - Sizes verified using by running through 1% sybr-safe gel
- Transformation of Rhizobia using conjugation
  - Planning
    - pKT230 has origin of transfer but requires RP4 helper plasmid
    - Step 1: Transform RP4 into *E. coli* with pKT230 using conjugation
    - Step 2: Transform both plasmids into Rhizobia strains using conjugation
    - Plasmids: pKT230, pETcoco
  - Made 5 mL liquid culture of *E. coli* with pKT230 (LB + Kan) and 5 mL culture of *E. coli* with RP4
- Results from midi-prep and gel purification of K125000 yielded low concentrations
  - Decided K125000 was too unreliable – will focus mainly on pKT230 and pETcoco-2
  - Gel of BBa_K125000 from midi prep:



- Transformation of Rhizobia using chemical competency method
  - Started cultures of CIAT, Sm 356, 370, 371 to try CaCl2 method tomorrow

Tuesday 7/7

- Transformation of Rhizobia using CaCl2 method
  - Plasmids: pKT230 (kan selection), pETcoco2 (carb selection)
  - Strains: CIAT, 356, 370, 371
- Transformation of Rhizobia using electroporation
  - Plasmids: pKT230 (kan selection), pETcoco2 (carb selection)
  - Strains: CIAT, 356, 370, 371
- Transformation of Rhizobia using conjugation
  - Conjugatation of E. coli RP4 → E. coli pKT230
- Growth curve of Rhizobia strains using 96-well plate OD600 kinetic reader (previous assay did not work)
  - OD600 measured every 10 minutes for 48 hours
- Antibiotic 96 well plate assay
  - Strains: CIAT, 356, 370, 371
  - Antibiotics: kan, cam, rif, strep, tet
- Solid plate rifampicin resistance assay for Rhizobia
- Transformation of Cyanobacteria using natural method
  - Plasmids: pKT230, pETcoco-2
  - Strain: PCC 7002
- Amplification of FLP constructs for Cyanobacteria and Rhizobia



- Every component except the non-circular upstream homology arm of SM 1021.356 (lane G) was amplified
- Lane A is present in very low concentration; worth extracting anyways
- Will do a gel extraction of lanes A, C1, C2, E, G (where the DNA should be), I1, I2, K, M, and O tomorrow
    - C1/I1 is the upper band in each lane, and C2/I2 is the lower (brighter) band in each lane. C2/I2 should contain the kanR cassette.

Wednesday 7/8

- Transformation of Cyanobacteria using natural method
    - Plated
- Gibson Assembly of Flp constructs
- Analyzed results of Rhizobia growth and antibiotic resistance assays
- Tested growth of Rhizobia strains at different centrifugation speeds
- Designed and ordered primers to verify pKT230
- Transformation of Rhizobia using conjugation
    - Conjugation results for E. coli RP4 → E. coli pKT230:
        - 10^-4 dilution: 2 colonies
        - No dilution: 25000 colonies (10^4)
        - 2 uL mixed plate: 80 colonies
        - 30 min incubation time
        - Efficiency: 10^4 / 10^ 9 = 10 ^ -5
    - Checked that E. coli grows on TSB
- ELIC Experiment #1
    - Results: unsuccessful
    - Possible explanations
        - If GFP was still present in backbone, amilCP would not have been inserted efficiently.
        - Next time, perform gel purification of pZE21G to remove GFP.
        - amilCP primers not specifically designed for ELIC.
        - Poor electroporation time constant.

Thursday 7/9

- Transformation of Rhizobia using conjugation
    - Conjugation of E. coli pKT230 + RP4 → E. coli ECNR2
    - Plated resuspended in 250 uL and dilutions: 0, 10^-4, 10^-6
- Amplification and circularization of FLP constructs
- Transformation of Cyanobacteria using conjugation
    - Made ATCC1040+LB plates
- Verification of pKT230
- Purification of PCR products of CRISPR guide RNA
- 16S PCR to verify all strains

Friday 7/10

- Transformation of Rhizobia using conjugation
    - Results from conjugation of E. coli pKT230 + RP4 → E. coli ECNR2
        - No dilution: 46, 122, 121 colines
        - 10^-4 dilution: 0 colonies
        - 10^-6 dil: 0 colonies
        - Streaked control: 1 colony on mixed
        - Efficiency: 10^-7
        - oriT compatible with RP4, can be transferred
- ELIC Experiment #2
    - Results: unsuccessful
        - Vector contamination – sfGFP is still being expressed
        - Most likely reason: first EcoRI site does not exist. EcoRI digest is not cutting sfGFP out.
        - Solution: retry experiment with new primers for KpnI/HindIII
- Ran 16s on all four rhizobium strains, 7002 stock, and a rhizobium strain that had lawn growth on an antibiotic it should not have been resistant to.
- Results from Rhizobia growth/antibiotic resistance assays: no growth
    - Possible explanations: dead cultures, not long enough growth time
    - Remeasure plates later; set up new growth assay to go over weekend – longer time

WEEK 7 (7/13 to 7/19, 2015)

Colin - 7/13/15

PCR OF FLP CONSTRUCT COMPONENTS: ATTEMPT 2

Tube	KAPA	Forward Primer	Reverse Primer	Template	2.2 M Betaine
A	20 µl	277 (0.5 µl)	278 (0.5 µl)	PCC 7002 Genome 4.5 ng/µl (3.0 µl)	16 µl
B	20 µl	277 (0.5 µl)	278 (0.5 µl)	n/a	19 µl
C	20 µl	279 (0.5 µl)	280 (0.5 µl)	pZE21G 24.4 ng/µl (1.0 µl)	18 µl
D	20 µl	279 (0.5 µl)	280 (0.5 µl)	n/a	19 µl
E	20 µl	281 (0.5 µl)	282 (0.5 µl)	PCC 7002 Genome 4.5 ng/µl (3.0 µl)	16 µl
F	20 µl	281 (0.5 µl)	282 (0.5 µl)	n/a	19 µl
G	20 µl	313 (0.5 µl)	314 (0.5 µl)	SM1021.356 Cells (colony)	19 µl
H	20 µl	313 (0.5 µl)	314 (0.5 µl)	n/a	19 µl
I	20 µl	315 (0.5 µl)	316 (0.5 µl)	pZE21G 24.4 ng/µl (1.0 µl)	18 µl
J	20 µl	315 (0.5 µl)	316 (0.5 µl)	n/a	19 µl
K	20 µl	317 (0.5 µl)	318 (0.5 µl)	SM1021.356 Cells (colony)	19 µl
L	20 µl	317 (0.5 µl)	318 (0.5 µl)	n/a	19 µl
M	20 µl	319 (0.5 µl)	314 (0.5 µl)	SM1021.356 Cells (colony)	19 µl
N	20 µl	319 (0.5 µl)	314 (0.5 µl)	n/a	19 µl
O	20 µl	317 (0.5 µl)	320 (0.5 µl)	SM1021.356 Cells (colony)	19 µl
P	20 µl	317 (0.5 µl)	320 (0.5 µl)	n/a	19 µl
Q	20 µl	n/a	n/a	n/a	20 µl

Danny - 2015/07/13

Conjugation

Conjugation #	Donor	Recipient	R-Select	D-R SelectI
A	#873	#795	Cam	Cam+Kan
			SDS	SDS+Kan
E	#874	#796	SDS	SDS+Kan
F	#874	#797	SDS	SDS+Kan

- grow 2mL cultures of donor and recipient strains ~~for 9-10 hours in LB containing the appropriate antibiotics~~ to an OD of ~1.0
  - Cultures started at 8:45am in SDS or kan from overnight cultures
  - Ready at ~16:30

- Take out plates to conjugate on and let them prewarm in incubator; leave them slightly uncovered, so if wet they will dry
- wash and concentrate cells
- Note: the goal of this step is to remove antbiotics from the surface of the cells and re-suspend them in a small volume so they are maximally concentrated for efficient cell-cell contact
    - transfer 1.9mL of culture to a centrifuge tube (all 3mL for all) - x
    - Centrifuge 13,500 rcf, 1 min, room temp - x
    - Remove supernatant - x
    - Resuspend in 1mL LB - x
    - Centrifuge 13,500 rcf, 1 min, room temp - x
    - REmove supernatant and resuspend in 1ml LB - x
    - Centrifuge 13,500 rcf, 1 min, room temp - x
    - resuspend in 150uL LB min -
    - If I do a 10:990 dilution in a cuvette and check OD600, this should read 0.2.
        - Donor - 1:100 dilution is OD of .083
            - Already in conj mixture, so can't do anything about that
    - combine donor and recipient cultures at 10:10uL ratio

  - pipette donor: recipient mixture on the surface of an LB plate (no antibiotics) as a series of 2x 20 ul, 6x 10 ul spots so they can conjugate on a solid substrate
  - incubate plate at 37C for 1 hour, in at 18:10 out at ___ (this is the upper incubator in 209)
- re-suspend cells directly off plate in 2x750mL washes of LB over the plate surface
- Concentrate to 250uL
- Plate onto selection plates
    - create dilution series of cells using 1-step dilutions: E+0, E+1, E+2, E+3, E+4,… E+6
    - Make dilutions in a 96-well plate, do 20:180uL.
    - Plate 50uL, plate -5
- plate dilution series on two sets of plates:
  1. plates that select for recipient
  2. plates that select for recipient that has received DNA by conjugation

Erin – 7/13/15

- Made, autoclaved, and aliquoted 2xYT broth
- Started 5 mL liquid cultures of pZE21G and pKT230 for miniprep tomorrow.
- Made new ELIC primers using KpnI and HindIII sites.
- Wrote up ELIC protocol on OneNote under Protocols > ELIC.

Holly - 13JUL15

Results from Friday:

No growth in centrifugation experiment
Chemical transformation unsuccessful
Working stock: streak of CIAT is clear but plate shows contamination

ANTIBIOTIC PLATES

- Same results when streaked from master stock and working stock

STRAIN RESISTANCE				
	R. tropici CIAT	S. meliloti 356	S. meliloti 370	S. meliloti 371
Rif	NO	NO	NO	NO
Spec	YES	NO	NO	YES
Strep	NO	NO	NO	NO
Carb	YES	NO	NO	NO
Kan	NO	NO	NO	NO

ANTIBIOTIC 96-WELL PLATES

- First measurement (48 hours)
  - No growth on strep
  - Growth of some CIAT in Rif wells
- Second measurement (72 hours)

STRAIN RESISTANCE (Second measurement)				
	R. tropici CIAT	S. meliloti 356	S. meliloti 370	S. meliloti 371
Rif	MAYBE	NO	NO	NO
Spec	YES	NO	NO	NO
Strep	NO	YES	YES	YES
Carb	NO	NO	NO	NO
Kan	NO	NO	NO	NO

Found electroporation protocols for tomorrow

Made new working stock of CIAT

Made 3 sets of 6mL cultures of each Rhizo strain for transformations and growth/antibiotic assay (will do tomorrow)

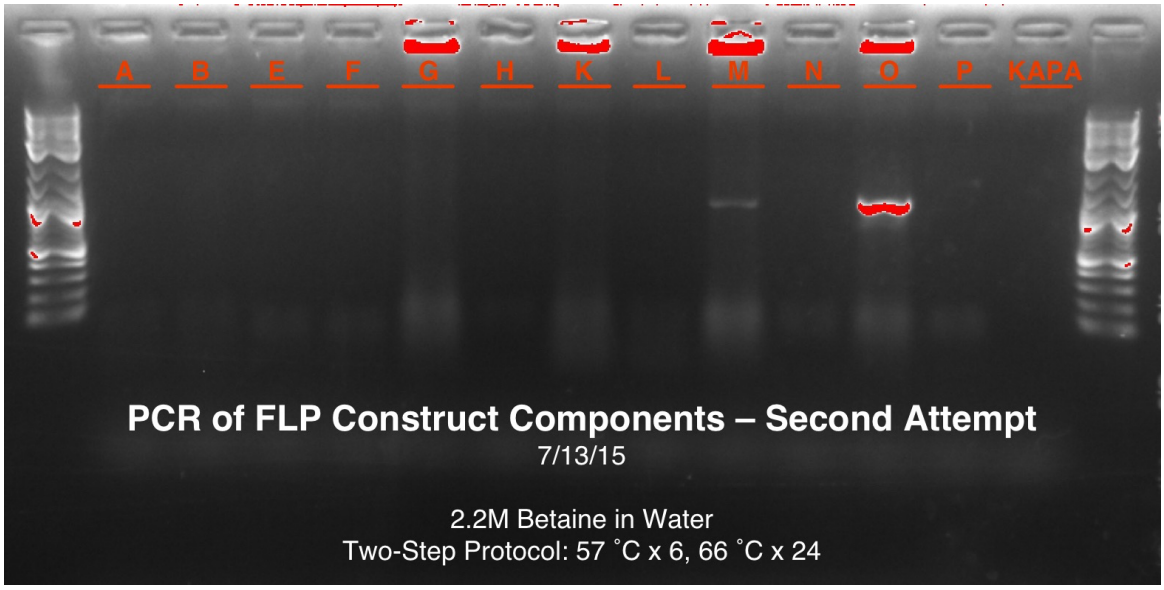
Made aliquots of TSB broth

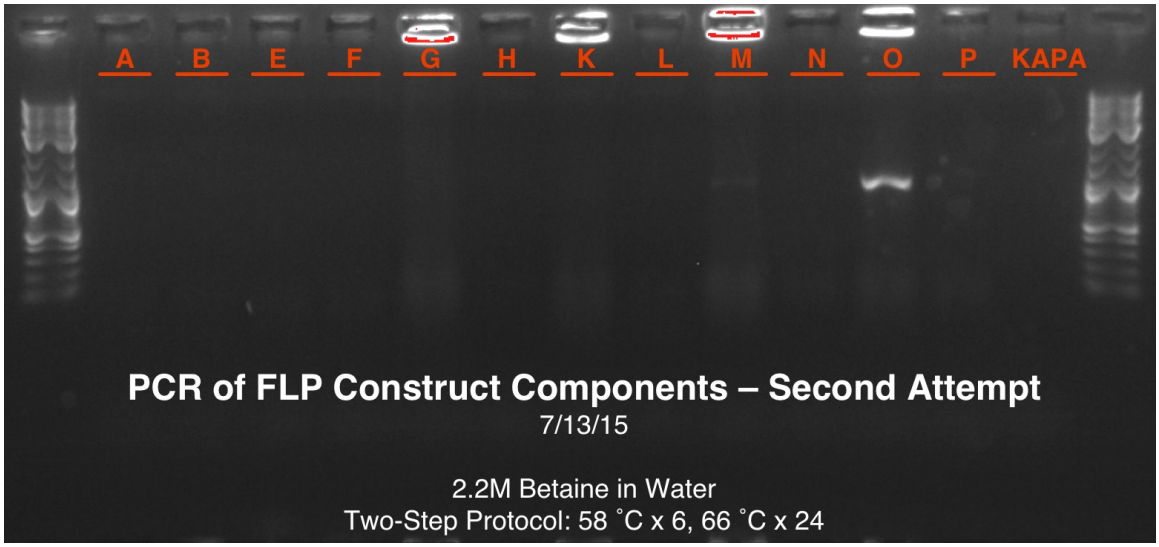
Centrifugation experiment using culture from 10JUL15

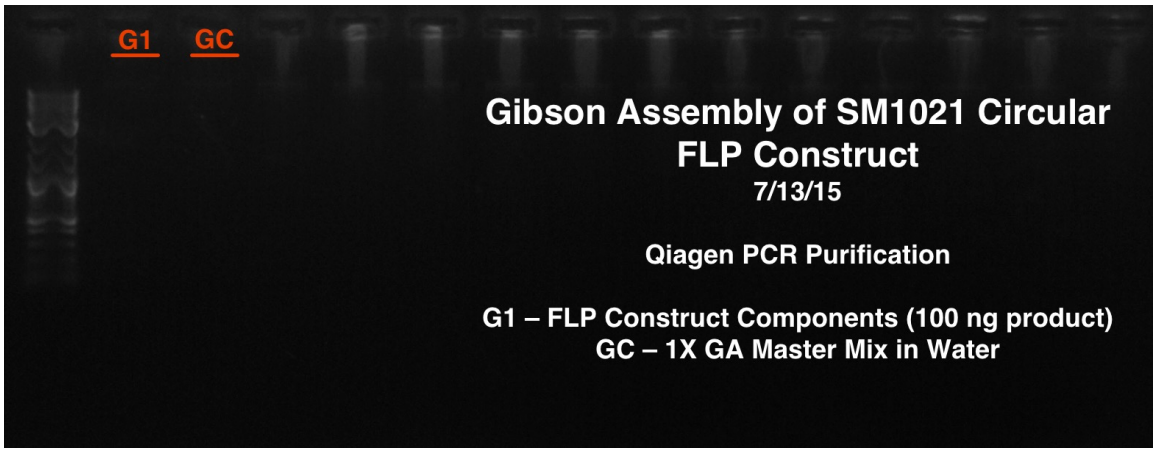
Colin - 7/14/15

GEL OF YESTERDAY'S PCR AND GIBSON

- 1 % agarose, 40 min, 150 V, 400 mA, 1x SybrSAFE (all on the same gel)







- Nuclease seems to be present
- Did not amplify samples C, D, I, and J because we are out of pZE21G to use as template and last week's PCR yielded plenty of kanR cassette

CYANO MEDIA ISSUES

- Made more ATCC 1047 medium
- Kan Resistance:
  - Cyano may appear to be resistant to kan because kan is unstable at high pH's. Since ATCC 1047 is at pH 8.5, the antibiotic may degrade before acting on the cells.
  - Danny found a protocol that using kan at 100 µg/ml (3.3x E. coli concentration) when culturing cyano

- Kan resistance assay on plates:
  - PCC 7002 was plated on pH-balanced ATCC 1047-Kan plates with varying levels of antibiotic:

Plate	Kan Concentration (µg/ml)
A	0
B	30 (1x E. coli)
C	60 (2x E. coli)
D	90 (3x E. coli)
E	120 (4x E. coli)
F	180 (6x E. coli)

  - Plates were grown in 38 ˚C incubator
- Liquid-Only Culturing
  - Ordered LifeTech LIVE/DEAD cell stain kit
- 96-Well Plate Assay for Growth Curve and Antibiotic Resistance Assay
  - Antibiotics to Test:
    - Kanamycin (E. coli 1000x = 30 mg/ml)
    - Carb (E. coli 1000x = 50 mg/ml)
    - Tet (E. coli 1000x = 12 mg/ml)
    - Spec (E. coli 1000x = 95 mg/ml)
    - Strep (E. coli 1000x = 50 mg/ml)
    - Rifampicin (E. coli 1000x = 50 mg/ml)
  - Antibiotic Concentrations to Test (relative to standards for E. coli):
    - 0.5x
    - 1.0x
    - 2.0x
    - 4.0x
  - Media to Test:
    - ATCC 1047 pH 8.5
    - A+ (pH 8.2)
  - Antibiotics wells will be grown in ATCC 1047 pH 8.5
  - Solutions were prepared today; assay will be started tomorrow
    - Each well will contain 150 µl total liquid volume (145 µl media, 5 µl inoculation)

- Starter Cultures:
  - 4 ml saturated PCC 7002 was inoculated into 20 ml ATCC 1047 and left in the shaker overnight
  - Single colony of DH5a was inoculated into 5 ml LB and incubated overnight

		1	2	3	4	5	6	7	8	9	10	11	12
		0.5x	0.5x	0.5x	1x	1x	1x	2x	2x	2x	4x	4x	4x
A	Kan												
B	Carb												
C	Tet												
D	Rif												
E	Spec												
F	Strep												
G	ATCC 1047 pH 8.5	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
H	A+	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

- Characterizing E. coli growth at pH 8.5
  - Will be cultured in the same conditions as the plate above
  - Added 100 µl 1 M KOH per 10 ml LB min to make LB min pH 8.5
  - Strain to be used is DH5α

		1	2	3	4	5	6	7	8	9	10	11	12
		0.5x	0.5x	0.5x	1x	1x	1x	2x	2x	2x	4x	4x	4x
A	Kan												
B	Carb												
C	Tet												
D	Rif												
E	Spec												
F	Strep												
G	LB min pH 8.5	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
H	Cyano Media	ATCC 1047 pH 8.5	ATCC 1047 pH 8.5	ATCC 1047 pH 8.5	ATCC 1047 pH 8.5	ATCC 1047 pH 8.5	ATCC 1047 pH 8.5	A+	A+	A+	A+	A+	A+

POURING ATCC 1047 PLATES

- For Colin:
  - 8 x ATCC 1047 pH 8.5
  - 3 x ATCC 1047 pH 8.5 + 1x Kan
  - 3 x ATCC 1047 pH 8.5 + 2x Kan
  - 3 x ATCC 1047 pH 8.5 + 3x Kan (One of these is NOT pH-Balanced)
  - 3 x ATCC 1047 pH 8.5 + 4x Kan
  - 3 x ATCC 1047 pH 8.5 + 6x Kan

SOME FACTS

- Spec is BACTERIOSTATIC
- Strep is BACTERIOCIDAL

Danny - 2015/ 7/ 14

Goals:
Gibson assemble together promoters and citrine
Email Akos
Finish conjugation
Make ATCC 1047 plates

Emailed Akos!
PCR amplified PsaA, Cpc48, and isiAB with Dan Shapiros Plasmids. (290, 289, 305, 306, 299, 300)
Used Kappa hifi
20microL KHF
17.5 microL MilliQ
.75 microL forward primer
.75 microL reverse primer
1 microL template

Ran in 150mL TBE, 1.5g Agarose, 5 microL SyberSafe

No Bands whatsoever.

Ways to fix it:
Use Betaine instead of MilliQ (no milliQ!!)
Remake working primer stocks
Check primer sequences

Finished conjugation:
Washed Undiluted plate with ATCC without antibiotics
Centrifuged down at 13,500 rpm for six minutes (centrifuged down multiple times before but did not seem to work. Probably lost a significant amount of bacterium.)
Resuspended cyano in about 300 microliters
Made three dilutions. Plated triplicate plates of each
DID NOT PLATE UNDILUTED PLATES
Used 30 microliters of undiluted into two liquid stocks
Had issues due to messing up agar

Terrible terrible time I hate everything.
Possible used 30microliters per plate instead of 50
Ways to fix it:
Use Filter
Use methylase helper plasmid
DON'T mess up agar
Use proper measurements
Centrifuge properly
Use dilutions of 10 to -2 10 to -4 and 10 to -6
Centrifuge properly. Should we do a centrifugation assay?

Holly - 14 Jul2015

- Plated Rhizo conjugations (12 hr incubation)
- Rhizo antibiotic resistances (plate assay)

	CIAT	356	370	371
Rif	•	•	•	-
Spec	+	•	•	+
Strep	•	•	•	-
Carb	+	•	•	-
Kan	•	•	•	-

- Rhizo conjugation plating
  - Dilutions: 0, -2, -4, -6
  - Resuspend in 250 uL TSB (After aspirating and centrifuging)
    - C & D in 250 uL TSB + colicin
  - Plate 50 uL each
    - For recipient controls + no dilutions: added 100 uL TSB
- E coli conjugation results
  - Controls:

	Cam	Tet + cam	Kan +cam
D conrol	•	•	-
R control		•	-

Jessica - 14JUL15

Results:
All results were lawns

SPEED	TIME	R. tropici CIAT 899	S. meliloti 356
10000 rpm	**1 min**	LAWN	LAWN
8000 rpm	**10 min**	LAWN	LAWN
455 rcf	**10 min**	LAWN	LAWN
10000 rpm	**5 min**	LAWN	LAWN
9820 rcf	**10 min**	LAWN	LAWN
Control	**—**	LAWN	LAWN

Cultures from 13JUL15 not ready for growth assay or transformation

Made 4mL cultures of every Rhizo strain for Lionel

Colin - 7/15/15

PCC 7002 LIQUID ANTIBIOTIC AND GROWTH ASSAY

- Two 96-well plates were set up as described in 7/14
- 5 µl of overnight culture of PCC 7002 or DH5a was inoculated into 145 µl of each well solution
- Enough of each media sample was made to repeat the experiment if needed; media samples are in fridge
- Created a protocol on the BioTek EON plate reader (little one controlled by laptop in Isaacs lab) for reading both OD730 and OD600 of the plates
    - Shake (orbital, 380 rpm) for 30 seconds
    - Read OD730 of every well
    - Read OD600 of every well
    - Export files in Excel
- A plate read was done at 0h, before incubation
- Both plates are in 38 ˚C shaking incubator (shaking at 215 rpm)

KANAMYCIN PLATE ASSAY IN CYANO

- Will put off until tomorrow at the latest in anticipation of getting finding a good lighting solution to the CO_2 incubator

WORKING WITH THE FLOW CYTOMETER

- E-mail Ken to let him know when we would need it
- Files are saved to "DATA" folder, which then saves to a server; need a dongle from the Isaacs lab to get access to the files.
    - How do you access the server?

GENOME EXTRACTION OF 7002

- For re-PCRing all FLP components
- Obtained 100 µl of good quality DNA at 7.3 ng/µl

RE-PCRing FLP COMPONENTS FROM SCRATCH

- Made fresh working stocks of all primers (20 mM) to use in PCR tomorrow
- Will try both betaine and water
- Need to obtain fresh SM1021 cells for template… or try
- Need to obtain KAPA mix

Danny - 2015/7/15

Goals: Check the primers for the cyano promoters
Get primers for cyano promoters in general (nirA up in this)
Finish primers for T1 terminator
Secure pRL623 plasmid from SOMEONE.
Pray fervently for successful conjugation
Make Primers for Crispr project
Look into finding Co2 bubbler. Can we use fish tank bubblers?

Found a fire stone valve/ bubbler apparatus from Dan. This will work over fish tank bubblers
Can use CO2 line in tissue room
Started stocks of E. Coli with pKT230 for minipreps for an attempt at natural competency.
Designed primers for CRISPR project.
Plan on making a Gibson construct of
Cpc promoter, Target sequence, Guide RNA sequence, t 7 terminator, synthetic XbaI site, nirA promoter, Cas9, rrnb1 terminator and then using Elic to insert that construct as a whole.
Primers are finished, with proper homology arms for Elic.
Plan for when primers are in: Amplify all components of construct. Gibson assemble everything. Elic into KT230. Electroporate into DH5Alpha. Wait until we actually have a functioning transformation protocol for Cyano.

Erin - Wed. 7/15/15

- Made frozen stock of C31GIB.ΔλRed [pZE21::sfGFP]

Potential Collaborations:

- Columbia - lactobacillus

Gibson Assembly primers for BamHI/T1 terminator on pZE21G

	Sequence	Tm
Forward:	ACTCTCGGCATGGACGAGCTGTACAAGTAAaagcttgatgggggatcc	59.41
Reverse:	CTCGCCGCAGCCGAACGCCCTAGGTCTAGGgcggcggatttgtcct	60.48

ELIC primers for pKT230

Holly - 15Jul2015

- Grew up fresh Rhizo liquid cultures
- Analyzed E. coli conjugation results (see google drive)

Jessica - 15JUL15

Cultures from 13JUL15: CIAT and 371 are confluent but 356, 370 are barely thicker than clear… dead cells? Going to use anyways

96 well plate growth assay:
Used cultures from 13JUL15
Started at 2pm
30 degrees kinetic OD600 for 26 hours, 10 minute intervals

Transformation of Rhizobial cells:
Used culture from 14JUL15 (originally made for Lionel)
Protocol followed:
Electroporation in Rhizobia

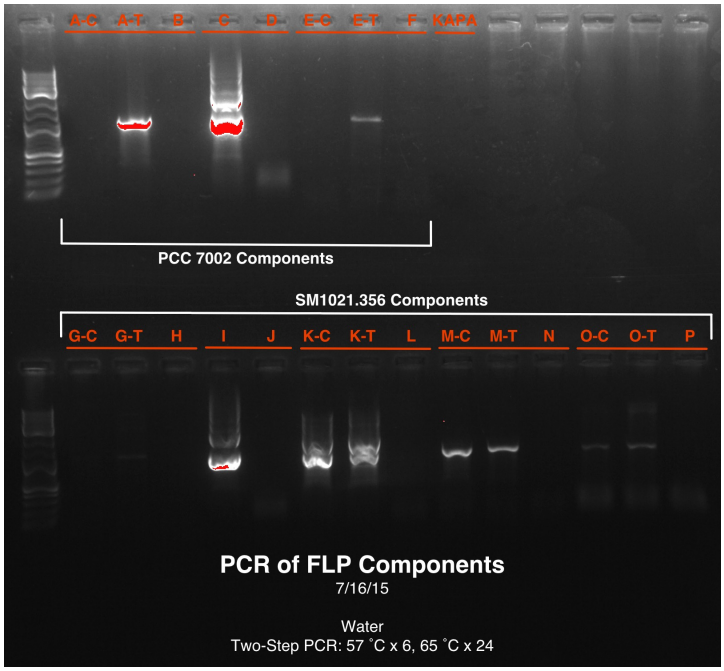
1. Grow liquid cell cultures in the shaking incubator (30C) until OD 600 is ~0.7.
2. Spin down 1 mL of the culture in a centrifuge at 10000 rpm for 1 min
    a. Literature recommends: 8000 rpm for 5-10 min at 4C
    b. For larger pellet: Use 3 mL of culture instead of 1 mL; centrifuge twice.
3. Remove supernatant, resuspend in 1mL milliQ H2O
4. Repeat steps 2-3; on the third centrifugation, resuspend in 50 uL milliQ H2O
5. Add 1ng/uL plasmid. For negative controls, do not add plasmid.
6. Transfer the DNA cell mix to a pre-chilled 1 mm gap electroporation cuvette. Keep the cuvette on ice
7. Electroporate at 1.8kV, 200 ohms, and 25 mF
    a. Literature recommends (for agro): five electric pulses. Electroporation was carried out with an electric pulse of 20.0 kV/cm and pulse length of ~10 ms (Cell-Porator® by Life Technologies). ~15 seconds between pulses, 0.15 cm electrodes with 25 uL cell culture
8. Add 1 mL of the recovery media (TSB) to the cuvette and transfer the mix to a recovery tube
9. Recover for 4 hours or overnight.
10. Transfer contents to a microcentrifuge tube and spin down at 10000 rpm for 1 min. Remove supernatant and resuspend in 100 uL of TSB.
    a. Have also tried: resuspending at 5000 rpm for 3 minutes
11. Plate cells on antibiotic plates. Spread them with glass beads and dry the plate with the lid slightly open in the incubator for ~5 minutes.
12. Incubate for at least 2-3 days and record growth each day.

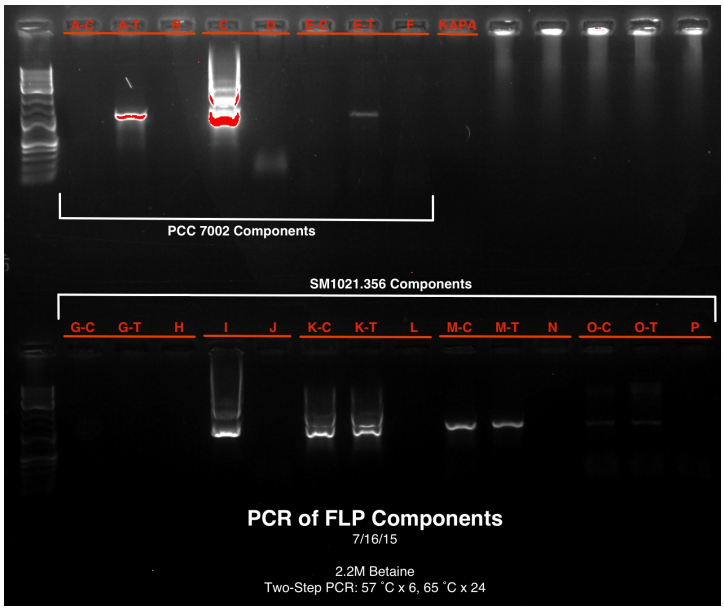
Colin - 7/16/15

RE-PCRing FLP COMPONENTS FROM SCRATCH

- Reaction will be run with 2.2M betaine and water in parallel
- Two-step procedure: 57 ˚C x 6, then 65 ˚C x 24
  - 57 ˚C seemed to work best based on the PCR attempted earlier in the week
- MISTAKE: Tubes C and D in betaine are in the water row, and tubes C and D in water are in the betaine row. Tubes will be switched back before running the gel.

- Gels:





- There appears to be no significant difference between nuc-free water and 2.2 M betaine in the reaction mixture… Maybe there will be a difference in the concentrations of DNA extracted. If anything, water seems to work better (see G-T band on water gel).
- Likewise, there seems to be no significant difference between using cells and total DNA as template. Cells seem to perform slightly better, but that might be because they were present in higher concentration than the genomic DNA.

CYANO CULTURING ISSUES

- Went to a hydroponics store to buy a large fluorescent lamp array to hang above the shaking incubator… This will free up the LED lamp for the CO_2 incubator (at least for long enough for us to get better lighting for the CO_2)

PCC 7002 LIQUID ANTIBIOTIC AND GROWTH ASSAY

- It seems like the 38 ˚C incubator causes condensation on the film seal of the plate, which affects the OD reading. Shaking the plate vigorously to get rid of the condensation seems to work, but that's a bit sloppy. Might retry assay next week with non-sealed plates if data comes out poorly this time.

Danny - July 16, 2015

Goals:
Buy lights for better cyano growth
Make growth assay/ urea sensitivity assay

Colin bought t5 6400k wavelength growth bulbs and a tekfour lamp from a nearby hydroponics store.

Erin - Thurs. 7/16/15

Well, accidentally used EcoRI instead of KpnI/HindIII…
Growing up 5 mL of pZE21G to miniprep tomorrow and try ELIC again.
Plated MG1655 and C31GIB.ΔλRed [pZE21::sfGFP].

Holly - 16Jul2015

- Colicin C (into 356) contaminated with fungus
- Control streak growth:

	D	R
kan	+	356, +
Spec + kan	•	371, +
Spec	•	371, +
TSB	+	356, +
TSB	+	370, +
Kan	+	370, +
Carb	+	CIAT, +
Carb + kan	+	CIAT, -

- Conjugation results saved on gDrive + Excel
- 16s for conjugation colonies
    - Purpose: to determine which species is the large colony and which is the small colony on the conjugant plates
    - Procedure:

Used Natalie's formula to calculate how much of each component to put in PCR tube (20 uL total):

1 uL template
940 primer F
941 primer R
KAPA fast
dH2O

The template comes from:

Use a clear, v-bottom plate and in each well (only using 2 rows) pipette in 20 uL nuc free h2o; pick from single colonies (8 large, 8 small) *used the C, no dilution plate (d --> 356, colicin + kan)

The PCR parameters used were: 95C for 3 min, 95C for 15 sec, 58 C for 15 sec, 42C for 1 min (repeat underlined 26x), 72C for 5 min, 10C infinity

Gel setup: (4 uL from PCR tube)

Ladder	**F01**	**G01**	**F02**	**G02**	**…**	**Ladder**
2uL	(Big)	(Small)	etc			

e. Set the gel at 120 V for 30 min
f. To submit for 16s sequencing: transfer remaining contents from the PCR tube (16 uL) into labeled PCR tubes; store at 20C before mailing (ordering form on GeneWiz)

    - Notes: noticed strange artifacts on the gel
        - Bands faint
        - Save the imaging file as SCN file, export as jpeg

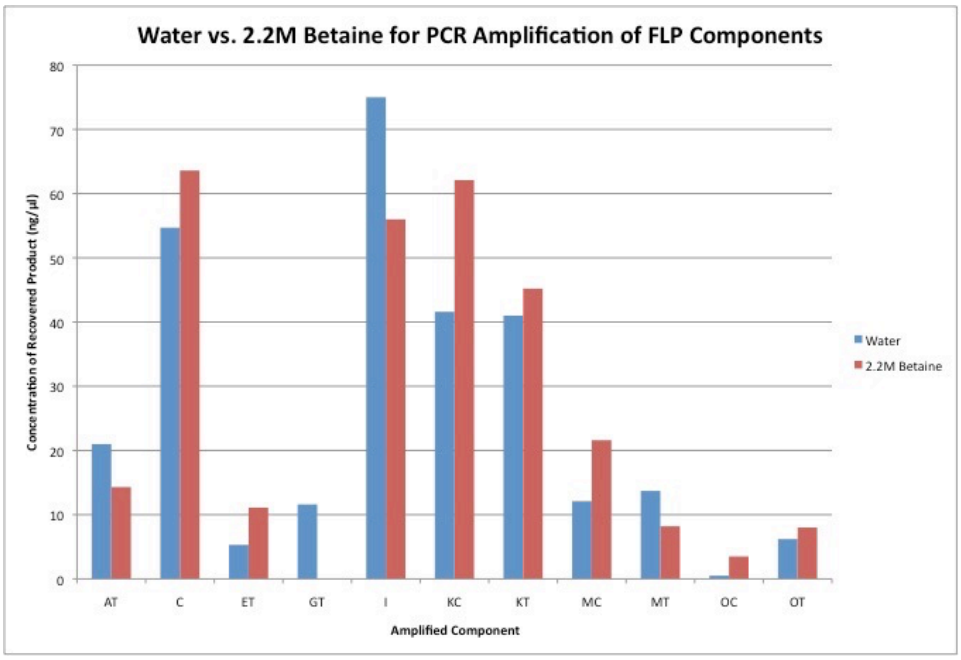
Colin - 7/17/15

BUILDING FLP CONSTRUCTS

- Gel extraction was done of yesterday's PCR products. Bands extracted: WAT, WC, WET, WGT, WI, WKC, WKT, WMC, WMT, WOC, WOT, BAT, BC, BET, BI, BKC, BKT, BMC, BMT, BOC, BOT
- Eluted into 30 µl warm buffer EB

- Quantification:

Sample	Concentration (ng/µl)
WAT	21.0
WC	54.7
WET	5.3
WGT	11.6
WI	75.0
WKC	41.6
WKT	41.0
WMC	12.1
WMT	13.7
WOC	0.5
WOT	6.2
BAT	14.3
BC	63.6
BET	11.1
BI	56.0
BKC	62.1
BKT	45.2
BMC	21.6
BMT	8.2
BOC	3.5
BOT	8.0



- What is the relationship between solution used and product recovery?
    - There seems to be a rough relationship between the solution used and the amount of product recovered… If you use one solution (either water or 2.2M betaine), there's a good chance that you will recover at least 50% of the same product by using the other solution.
    - The betaine samples tended to be of slightly lower quality (more RNA/protein contamination). This is something to be aware of in future experiments.

TYPICAL WORKFLOW FOR ASSEMBLING CONSTRUCTS

- Amplify components
- DPN1 digest/gel purify
- Gibson assemble constructs
- PCR amplify GA product with two outermost primers
- Gel purify amplified GA product
- **For circular constructs**: Re-GA amplified construct

GOALS

- Finish cyano light setup
- GA FLP PCR components
- GA Dan's promoters – let's do this over the weekend so that I take some time to look at Dan's notes and prepare everything
- PCR amplify GA product (if time permits)

GIBSON ASSEMBLIES AND PCRs

Sample	Component 1	Component 2	Component 3	F_Primer	R_Primer	
GC1	WAT	WC	WET	277 (384)	282 (385)	
GC3	WGT	WI	WKT	313 (319)	318 (320)	
GC5	WMT	WI	WOT	319	320	
GC6	BAT	BC	BET	277 (384)	282 (385)	
GC8	BMT	BI	BOT	319	320	

- PCR of Gibson-assembled samples – WILL RUN 7/19

- Protocol: GIBSFAST
  - 58 ˚C x 6, 65 ˚C x 24
  - Elongation steps @ 72 ˚C for 3.5 min

Erin - Fri. 7/17/15

- Miniprepped pZE21G (concentration _____)
- Put MG1655 and C31GIB.ΔλRed [pZE21::sfGFP] in deli fridge
- pZE21G ELIC protocol below - KpnI/HindIII digest, gel electrophoresis (2 bands), gel purification
  - Concentration of gel purified digested pZE21G was 3.5 ng/uL.

Step 2: Prepare Plasmid (pZE21G)
KpnI/HindIII Digest
Reaction Mix (40 µL total):

- 4 µL 10x CutSmart Buffer
- 2 µL KpnI
- 2 µL HindIII
- 25 µL DNA (pZE21G at _____ ng/µL for ~1000 ng total)
- 7 µL Nuc-free H_2O

Reaction Conditions:

- 37 deg C for 1 hr
- 80 deg C for 20 min (heat inactivation of HindIII, no heat inactivation for KpnI)

Grow up 5 mL MG1655 liquid culture in LB min broth.
Prepare SYBR Safe Gel

- Prepare a 1% agarose SYBR Safe gel while EcoRI digest is running. After the digest, you will need to immediately load the reaction into a gel and run it.
- Gel Mix: 75 mL TBE, 0.75 g agarose, 7.5 µL SYBR Safe. Add SYBR after microwaved solution has cooled.

Gel Electrophoresis

- Add 7 µL loading dye to each reaction.
- Load the gel with 3 µL ladder, 41 µL (total vol) digested pZE21G, and 41 µL negative control.
- Run at 150 V for 30 mins.
- Check bands under blue light.
- Cut out pZE21G band and proceed with gel purification.

Gel Purification

Follow Qiagen(?) kit instructions.

- Started 3 mL pZE21G for miniprep tomorrow.

Danny - July 17, 2015

Goals:

Set up Growth/ urea sensitivity assay

Redo Natural Transformation

Find Ava1 methylase sequence

DANNY'S GROWTH ASSAY

	1	2	3	4	5	6	7	8	9	10	11	12
A: Ni 7	Urea 50 mM	Urea 50 mM	Urea 50 mM	Urea 50 mM	Urea 100 mM	Urea 100 mM	Urea 100 mM	Urea 100 mM	Urea 150 mM	Urea 150 mM	Urea 150 mM	Urea 150 mM
B: Ni 5	Urea 50 mM	Urea 50 mM	Urea 50 mM	Urea 50 mM	Urea 100 mM	Urea 100 mM	Urea 100 mM	Urea 100 mM	Urea 150 mM	Urea 150 mM	Urea 150 mM	Urea 150 mM
C: Ni 3	Urea 50 mM	Urea 50 mM	Urea 50 mM	Urea 50 mM	Urea 100 mM	Urea 100 mM	Urea 100 mM	Urea 100 mM	Urea 150 mM	Urea 150 mM	Urea 150 mM	Urea 150 mM
D: no Ni	Urea 50 mM	Urea 50 mM	Urea 50 mM	Urea 50 mM	Urea 100 mM	Urea 100 mM	Urea 100 mM	Urea 100 mM	Urea 150 mM	Urea 150 mM	Urea 150 mM	Urea 150 mM
E: no NO_3	Urea 50 mM	Urea 50 mM	Urea 50 mM	Urea 50 mM	Urea 100 mM	Urea 100 mM	Urea 100 mM	Urea 100 mM	Urea 150 mM	Urea 150 mM	Urea 150 mM	Urea 150 mM
F												
G: A+ Growth Curve												
H: ATCC Growth Curve												

All put into a 96 well plate. NOT INOCULATED YET

145 microliters of solution

U = urea, milliMolar

Ni = nickel sulfate, micromolar

Holly - 17Jul2015

- Cultured 3 mL of rhizo for conjugation
- Conjugation results update:
  - Spec+kan streak: no growth donor, recipient growth
  - Sm371, E, 10^-4: 100s, single colonies
  - Stored conjugation plates at 4C
- Streaked rhizo on stronger TSB kan plates, stored in 29C incubator in Isaacs lab

- Did short streaks of conjugants (#7, #10--numbers based off of excel sheet of results): 356 kan + colicin, 370 kan + colicin
    - On stronger TSB plates (12 streaks about ~1cm long)

Lionel - July 18

1. Resuspend and make working stock (10uM) of GFP fix primers (361, 362)

<u>Gel Extraction of GFP with Overhangs and Gibson Assembly to promoter</u>

- Extracted DNA from gel for GFP with overhangs to nodABCD, bacA, melA, nifH, nodF, nodA
- Checked DNA concentration

Overhang to:	nodABCD	bacA	melA	nifH	nodF	nodA
Concentration (ng/ul)	109.8	30.9	58.0	33.3	10.5	55.4
Remarks					260/280 ratio was 2.4 - not great	

-Checked DNA concentration of extracted promoter DNA that Colin helped with

	bacA 1	melA 1	bacA 2	melA1	nodF
Concentration (ng/ul)	24.0	16.7	~20	~20	70.1
Remarks	*	*	*	*	Spectrum looks good

Poor spectrum (no identifiable peak at 260nm)

- Decided to go ahead with Gibson just for nodF

Used Ariel's spreadsheet to calculate volumes: Idea is to have a 1:1 ratio

- Diluted nodF and corresponding GFP fragment 1:5 in water
- Volumes: 10ul Gibson master mix, 3.82ul GFP, 4.72ul promoter, 1.46ul water
- 50C for 1 hour
- Then kept in -20.

<u>PCR of citrine with promoter and LIC overhangs</u>

- Template: pYU3034 (1ng/ul - Dan's dilution) for tube 1, pYU3034 (3ng/ul - my dilution from stock)
- Primers:
    - P5161, p5162
- Program: METHOD CALC || HOTLID 105, 30 || VOLUME 40 || TEMP 95.0, 300 || TEMP 98.0, 15 || TEMP 58.0, 15 || TEMP 72.0, 60 || GOTO: 2, 30 || TEMP 72.0, 300 || TEMP 12.0, 0 || END
- Used Hifi
- In each tube: 20ul KAPA Fast mix, 15ul 2.2M betaine, 2ul Forward primer (10uM), 2 Reverse Primer (10uM), 1ul Template
    - Ran 5ul of the PCR mix in a 1% gel

<u>PCR of sfGFP</u>

- Template: Natalie's plasmid, pZE21, pZA21
- Primers:

- 361, 362
- Program: METHOD CALC || HOTLID 105, 30 || VOLUME 40 || TEMP 95.0, 300 || TEMP 95.0, 15 || TEMP 58.0, 15 || TEMP 72.0, 240 || GOTO: 2, 6 || TEMP 95.0, 15 || TEMP 67.0, 15 || TEMP 72.0, 240 || GOTO: 6, 6 || TEMP 72.0, 300 || TEMP 12.0, 0 || END
- Used Hifi
- In each tube: 20ul KAPA Fast mix, 15ul 2.2M betaine, 2ul Forward primer (10uM), 2 Reverse Primer (10uM), 1ul Template
  - Ran 5ul of the PCR mix in a 1% gel
  - Notes: Extension time was twice what was needed

PCR of promoters

- Template: *S. meliloti 356* from working stock plate for the rhizo promoters. No template for Anderson promoters
- Primers:
  - MelA: p5164, p5165
  - NodF: p5166, p5167
  - BacA: p5171, p5172
  - Anderson Strong: p5173, p5174
  - Anderson Medium: p5175, p5176
  - Anderson Weak: p5177, p5178
- Program: METHOD CALC || HOTLID 105, 30 || VOLUME 40 || TEMP 95.0, 300 || TEMP 95.0, 15 || TEMP 58.0, 15 || TEMP 72.0, 240 || GOTO: 2, 6 || TEMP 95.0, 15 || TEMP 67.0, 15 || TEMP 72.0, 240 || GOTO: 6, 6 || TEMP 72.0, 300 || TEMP 12.0, 0 || END
- Used Hifi
- In each tube for rhizo promoters: 20ul KAPA Fast mix, 15ul 2.2M betaine, 2ul Forward primer (10uM), 2 Reverse Primer (10uM), Template (picked from colony)
- In each tube for Anderson promoters: 20ul KAPA Fast mix, 10ul 2.2M betaine, 5ul Forward primer (10uM), 5 Reverse Primer (10uM)
  - Ran 5ul of the PCR mix in a 2% gel
  - Notes: Lost track of order of tubes (though we can tell from the bands)

Expected fragment sizes
K125 MCS: 400bp
K125 Bsa: 530 bp (uncut); 170bp and 360 bp when cut
pKT MCS: 1150bp
pKT Bsa: 670bp (uncut); 210bp and 460bp when cut

Whole plasmid
K125: 9050bp
EcoRI, EcoRV, BsaI are one cutters
DraI is a two cutter: 2900bp and 6150bp
BsaI and BamHI: 650bp and 8400bp

pKT: 11800bp
EcoRI, BsaI are one cutters
BsaI and BamHI: 2850bp and 8950bp

TSB - may need higher Kan concentration
Use LB?
Or for TSB, use higher concentration. Try a few concentrations:

- Plate 1ml of cells
- Send Natalie pSMART sequence
- Send out annotated version of pKT230

Note: plac amplicon is 1.2kb, ptac amplicon is 1.9kb
Plac - Forward and reverse 69 degrees (for annealing region)

16S:
F (940): TGTAAAACGACGGCCAGTAGAGTTTGATCCTGGCTCAG
R (941): CAGGAAACAGCTATGACGGTTACCTTGTTACGACTT

Overlapping extension PCR
MelA (with overhangs): 240bp
BacA (with overhangs): 220bp
Lac (with overhangs): 1250bp
Citrine (with LIC overhang): 735bp

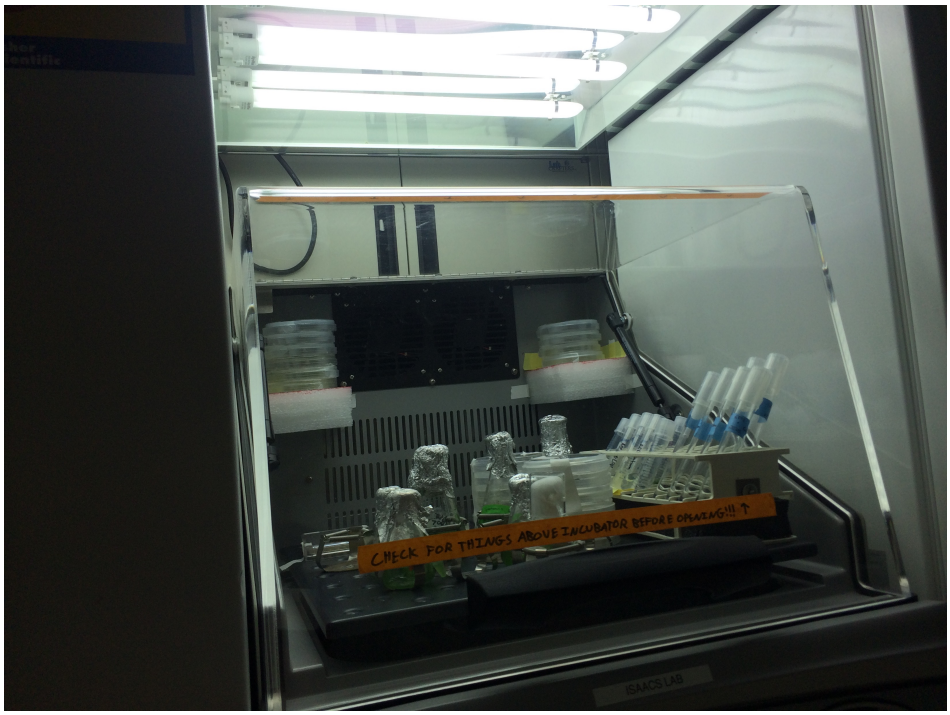
Colin - 7/19/15

ASSEMBLING DAN'S CONSTRUCTS

- Prepared today; will run tomorrow
- Will do a 1:5 dilution of all DNA components except #52 to get more workable concentrations

Sample	Component 1 (Concentration)	Component 2 (Concentration)	Notes
1	51 (pBAD_Citoverh, 114.8)	45 (Citrine_pBADoverh, 76.9)	pBAD-Citrine (K125)
2	52 (CI_Citoverh, 5.7)	50 (Citrine_Cioverh, 94.5)	Lambda-CI-Citrine (K125)
3	53 (Andsn_tetR_Rep, 53.3)	49 (ptet_Citrine, 142.9)	Anderson-tetR-Citrine (K125)
4	n/a	n/a	Control

LIGHT SETUP FOR CYANO SHAKING INCUBATOR



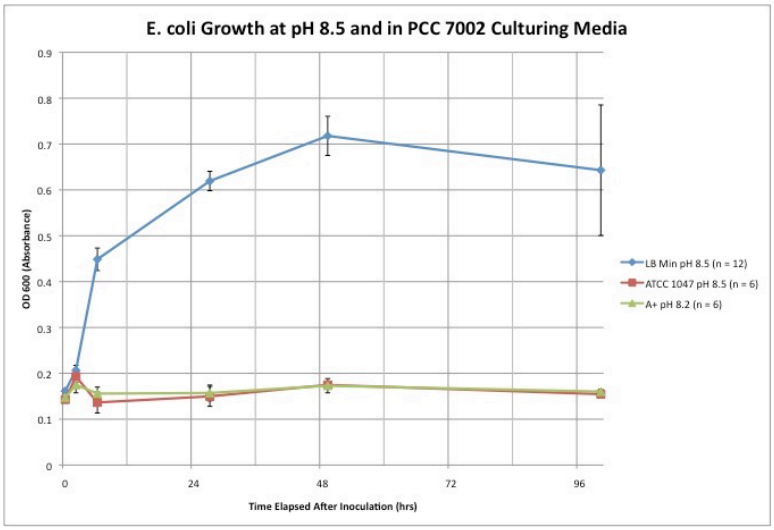
- Inoculated a fresh culture of PCC 7002 into ATCC 1047; can use to get another growth curve.
  - Starting OD730: 0.126
  - OD of this culture over time:

Time Elapsed Since Inoculation (hr)	OD_{730}
0	0.126
24	0.342
48	0.700
72	0.638
96	0.230

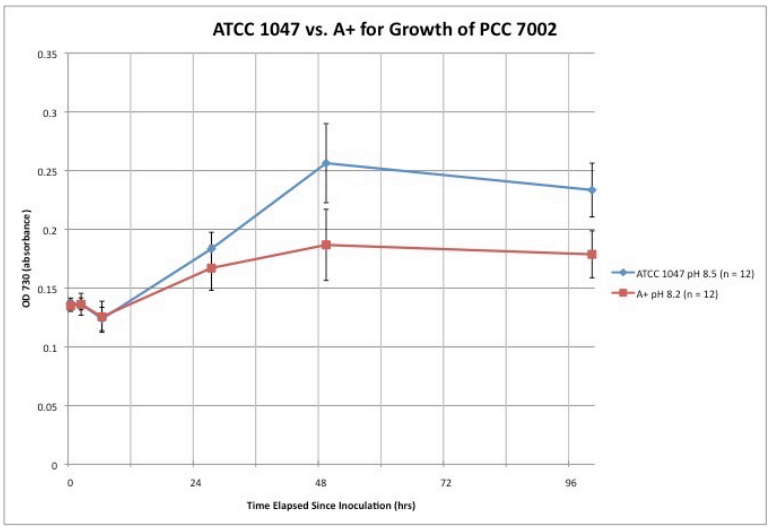
WEEK 8 (7/20 to 7/26, 2015)

MONDAY, 7/20

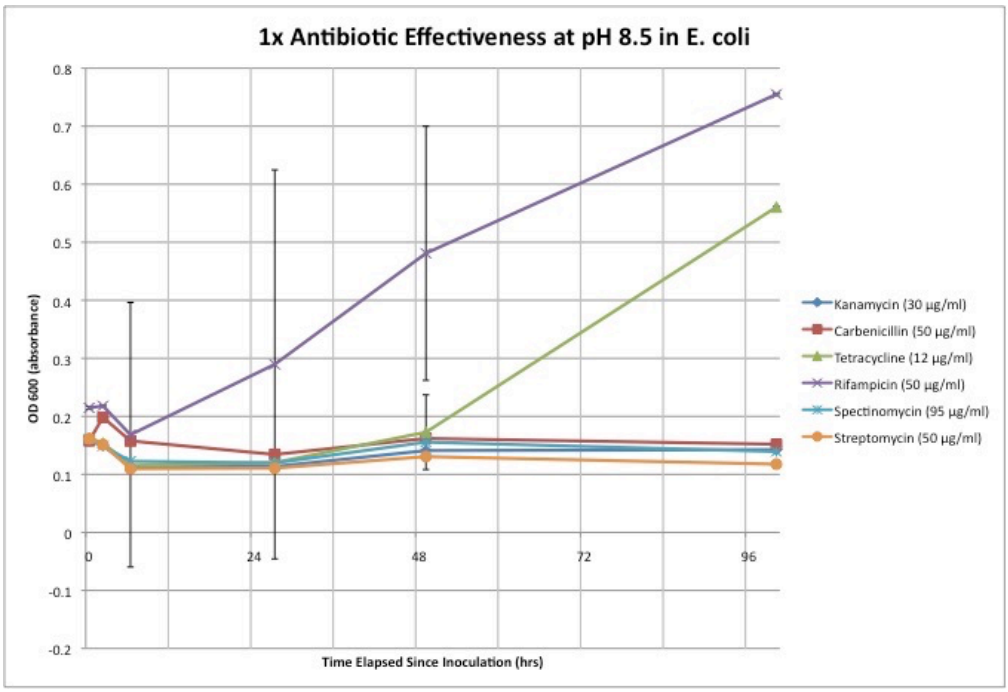
- General:
  - Transformed pCP20 (FLP plasmid with recombinase) into *E. coli* BL21 and MACH1
  - 50 uL of each sample was plated after a 2 hour recovery period
  - Site directed mutagenesis to knock out the BsaI cut site
    - Greater than 100 colonies for both pKT230 and k125000
    - Picked 17 colonies to screen by colony PCR
- Cyanobacteria:



  - E. coli does not grow in the media used to culture PCC7002, which means we will avoid some contamination issues.



  - PCC 7002 appears to grow more quickly in ATCC 1047 media, but the extent of growth in either sample is not really enough to make a conclusion on which leads to faster growth.



- All antibiotics tested are effective against E. coli in pH 8.5 media. This is a good indication that the antibiotics will not degrade when grown in A+ or ATCC 1047 media.
- Gibson assembled promoter-citrine constructs (pBAD-cit, Cl-cit, Anderson-ptet-cit); will amplify tomorrow
- Restarted the cyanobacteria growth assay
- PCR of the Gibson assembled FLP constructs and extracted some bands from PCC 7002 and Sm 1021; circularizing primers for Sm 1021 causes primer dimer and blurred PCRs

- Rhizobia:
  - Conjugants grew on all of the streaks with higher antibiotic concentrations; might choose to only work with CIAT and Sm 371 because they have antibiotic resistances
  - Did another antibiotic resistance assay: kan, spec, carb, tet, strep, cam

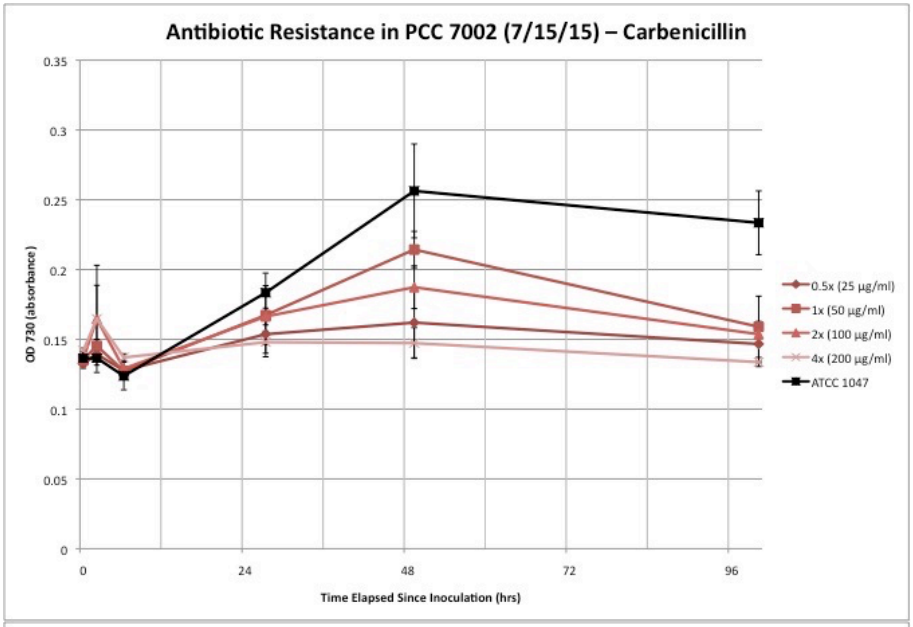
TUESDAY, 7/21

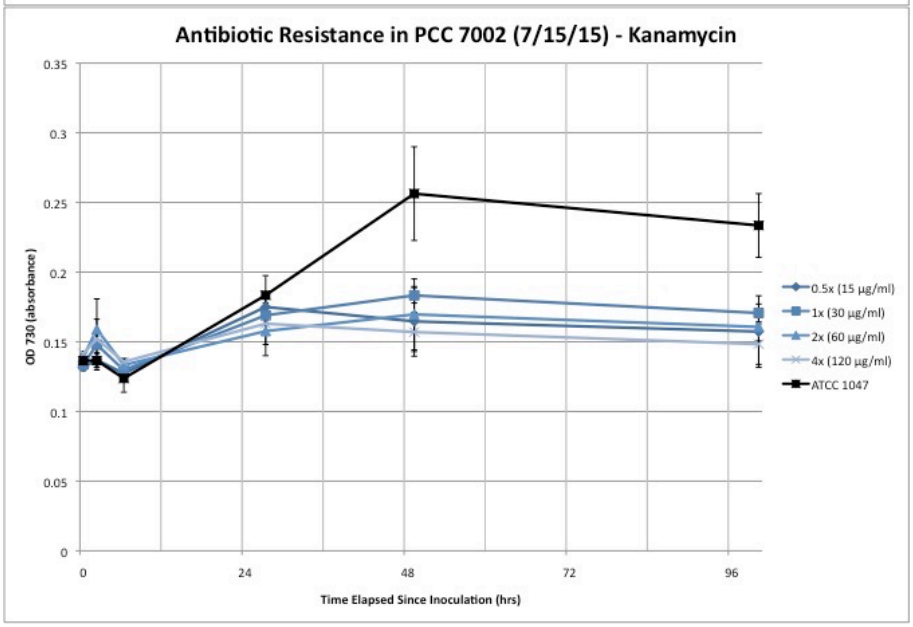
- General:
  - Results from transformation of pCP20 into MACH 1 and BL21

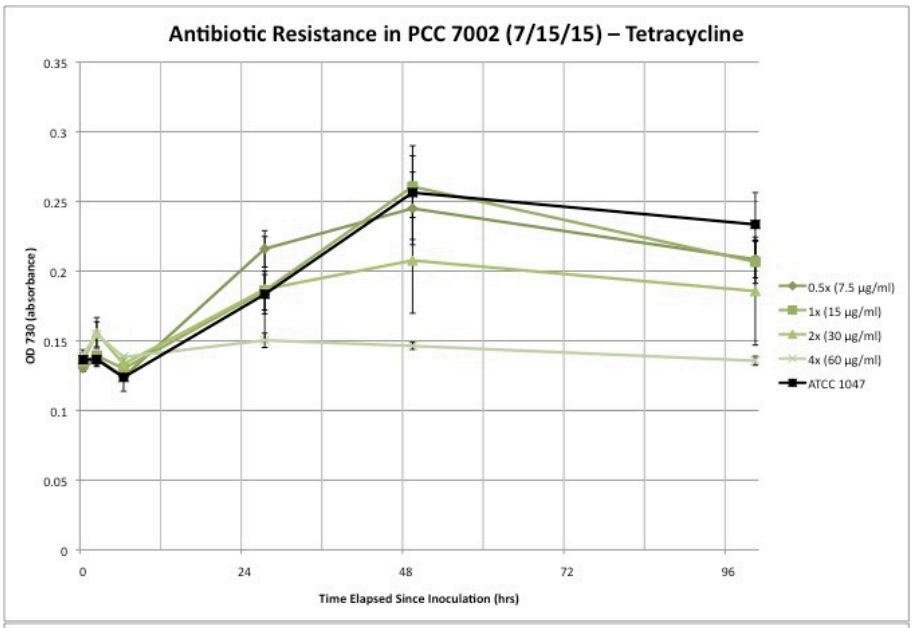
Sample	Description	Growth?
M1	MACH 1 + pCP20	No growth
M2	MACH 1 + pETcoco-2	Lawn
M3	MACH 1 control	No growth
B1	BL21 + pCP20	No growth
B2	BL21 + pETcoco-2	>1000 colonies
B3	BL21 control	4 colonies - Contamination?

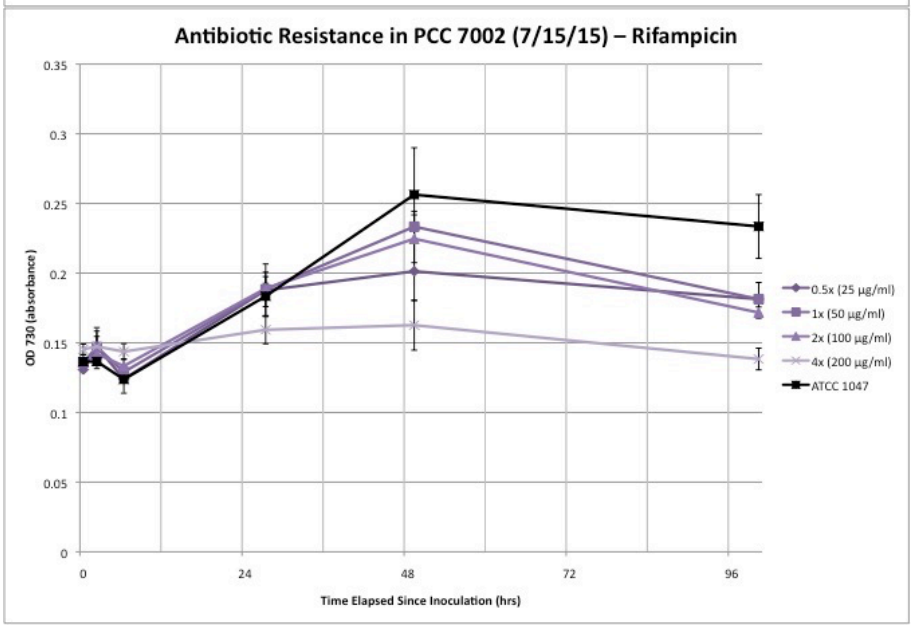
- Streaked a plate of DH10B [pkt230] and will make a glycerol stock out of this
- Miniprepped the k125000 clones and sent k125000 for sequencing
- Results of sequencing: unclear; many mismatches

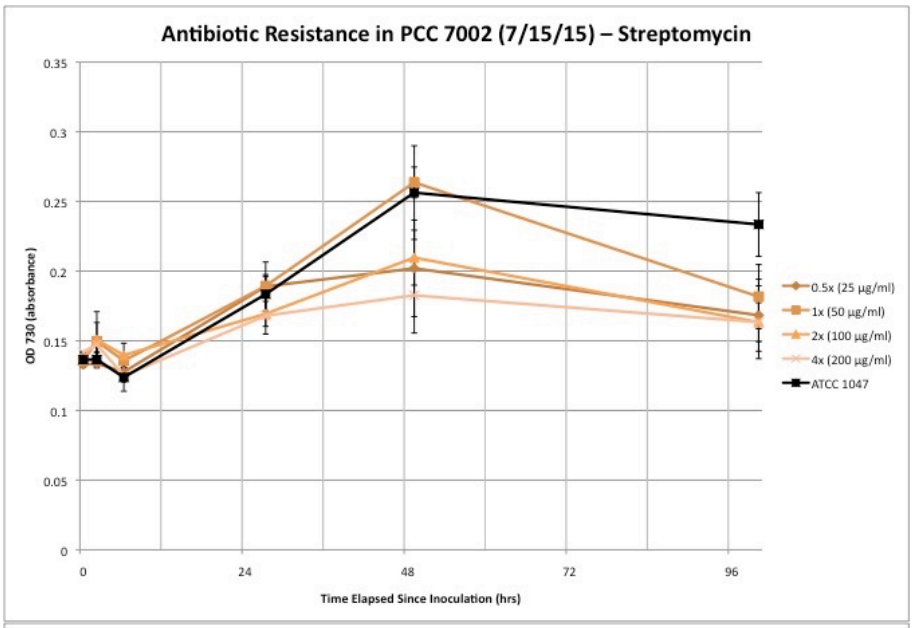
- Cyanobacteria:
  - Summary of antibiotic assays: almost every antibiotic effective at high concentrations (see below)

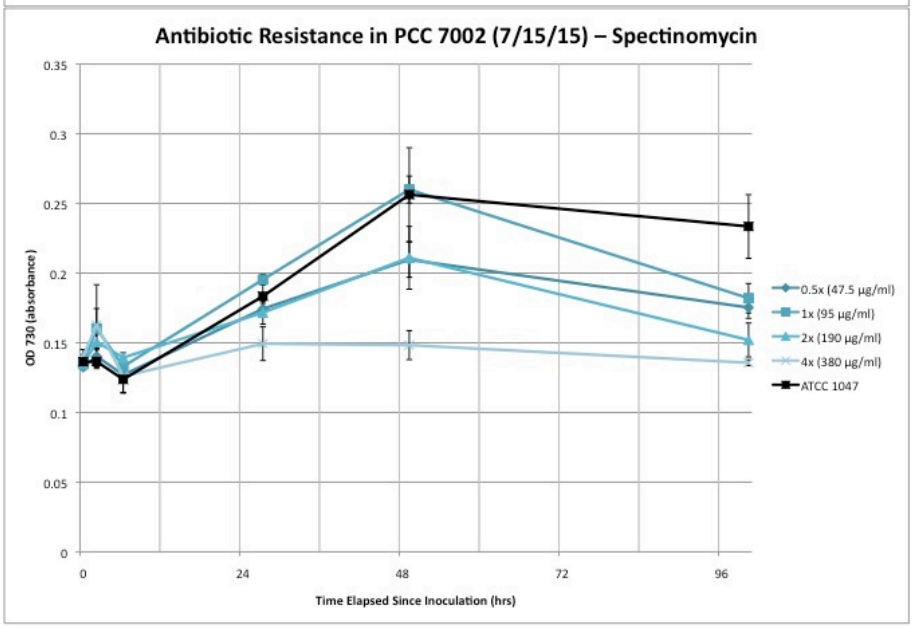





Antibiotic Resistance in PCC 7002 (7/15/15) – Tetracycline
OD 730 (absorbance)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
24
48
72
96
Time Elapsed Since Inoculation (hrs)
0.5x (7.5 µg/ml)
1x (15 µg/ml)
2x (30 µg/ml)
4x (60 µg/ml)
ATCC 1047


Antibiotic Resistance in PCC 7002 (7/15/15) – Rifampicin
OD 730 (absorbance)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
24
48
72
96
Time Elapsed Since Inoculation (hrs)
0.5x (25 µg/ml)
1x (50 µg/ml)
2x (100 µg/ml)
4x (200 µg/ml)
ATCC 1047





  - Looked at methods of using flow cytometry to analyze cell viability of cyanobacteria
  - Looked into CO2 incubator set-up
- Rhizobia:
  - 16s sequencing results from the conjugation indicated that there were *E. coli* contaminants
  - Electroporation of pkT230 and petcoco-2 into CIAT and Sm 371; using updated protocol with lower centrifugation speeds and multiple electric pulses
  - Restreaked *S. meliloti* strains from the Jacobs Wagner Lab

WEDNESDAY, 7/22

- General:
  - Made DH10B [pKt230] glycerol stock
  - Prepared pKT230 for sequencing with new reverse primer
  - Amplified citrine
  - Ran PCR to try to fix BsaI site in pKT230; not successful in amplifying band
- Cyanobacteria:
  - Did another PCR of Gibson-assembled constructs and adapted for LIC
  - Linear constructs amplified; circular ones unclear
  - Restarted the growth assay
  - Live/dead sorting protocol adapted from the LifeTech kit L-34856 (to see if we could obtain single genotypes using liquid-only culturing methods)
- Rhizobia:
  - Prepared for conjugation: made antibiotic resistant plates
    - Spec + tet (Recipient + helper plasmid)
    - Spec (R)
    - Kan + spec (shuttle vector + R)
    - Kan (shuttle)
  - DH5a[RP4][pkt230] = donor

THURSDAY, 7/23

- General:
  - Colony PCR of 17 colonies each for pKT230 and k125000
  - Digest with BsaI successful; streaked on plate so we can obtain single colonies
- Cyanobacteria:
  - Preliminary observations from flow cytometric analysis:
    - UTEX forms long chains of cells that are difficult to separate
    - The cells should be allowed to stain for longer than the recommended 15 mins
    - The cell samples were very messy. Of the 50,000 events recorded per sample, only about 5000 of them were gated as cells for both 7002 and UTEX. This may be because the 7002 cells were unhealthy, though they appeared to still be dividing. Many of the UTEX cells were still in long chain formations, so more vigorous resuspension conditions are required to fully separate individual bacteria.
    - Incubation of the cells with the stain in the dark is crucial.
- Rhizobia:
  - Created a glycerol stock of the conjugation donors strain
  - Conjugation of DH5a[RP4][pKT230] into CIAT and 371

FRIDAY, 7/24

- Cyanobacteria:
  - Finished the PCC 7002 flask growth assay; the strain did not grow in any of the conditions with antibiotic, and the ATCC and A+ controls barely grew
  - Will stick with A+ culture because there is less variability (ATCC media depends on seawater)
  - Planning to store some cultures in the Peccia lab CO2 incubator setup
  - Started new cultures of UTEX 2973 and PCC 7002 in BG11, ATCC, and A+ media

- Rhizobia:
    - Finished up the conjugation from earlier in the week
    - Results inconclusive because growth on controls

SATURDAY, 7/25

- General:
    - Ligation of LIC cassette + T7 into pKT230
- Rhizobia:
    - Started new antibiotic resistance agar assays
        - Resistances:
            - CIAT: spec (LB/TSB), carb (LB/TSB)
            - 356: kan (LB)
            - 370: kan (LB)
            - 371: kan (LB), spec (LB/TSB)
    - Optimization of PCR to amplify lac and tac promoters
        - Template: pYU2617

SUNDAY, 7/26

- Rhizobia:
    - 16s verification of *R. tropici*

WEEK 9 (7/27 to 8/2, 2015)

7/27

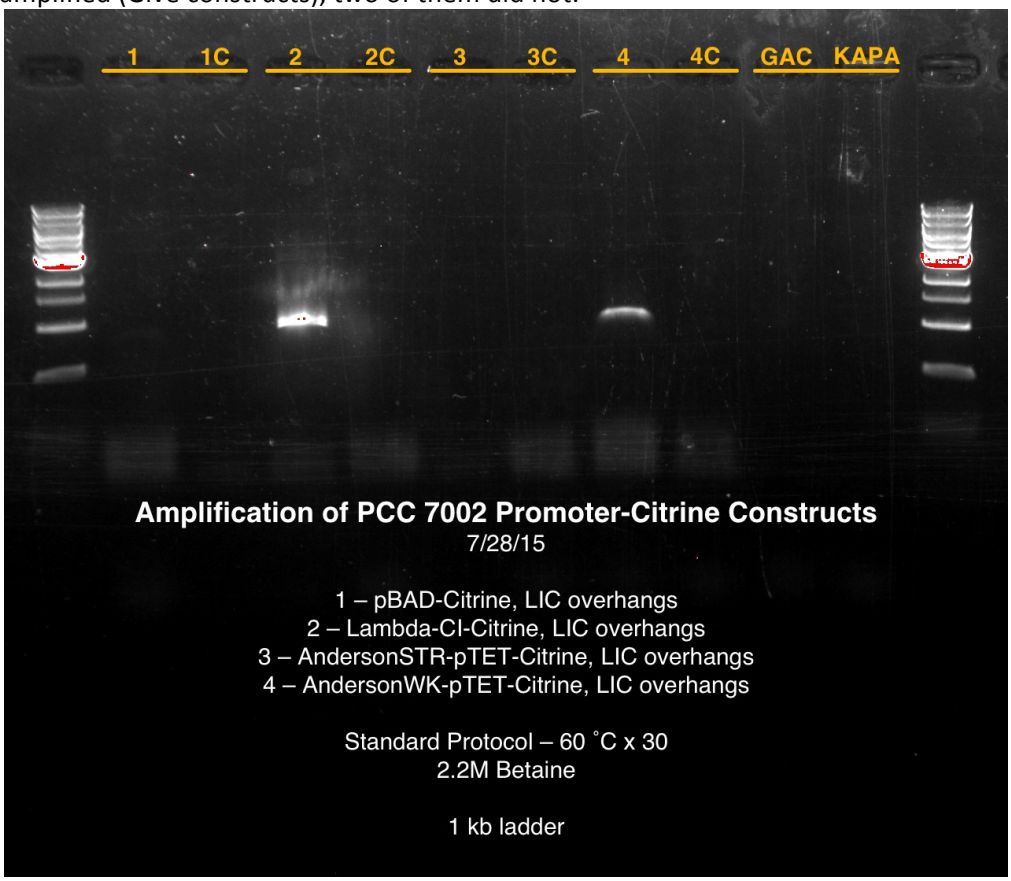
Colin: Colin ran growth cultures to check the effect of glycerol on 7002 growth, results showed faster growth/ higher peak OD

Danny checked the plated cyanobacteria from his attempt at natural transformation. The plates had lawn growth. Therefore, the lab had successfully plated and grown our strain of cyanobacteria. Danny also redid a failed PCR from week 8 using 2.2 molar Betaine instead of Nuclease Free water. Danny then plated the lawns of cyanobacteria onto selective media by both streaking and washing.

Lionel ran a ligation reaction to insert the LIC cassette into [pKT230]. Lionel also ran a PCR to confirm the electroporation of [pKT230] into R. tropici.

7/28

Colin analyzed growth cultures started last week built to test the effectiveness of various growth medias. Medium A+ showed no growth. This was most likely due to degraded vitamin B12. Colin ran a PCR of the constructs Dan had made two weeks ago. Two of them sucessfully amplified (Give constructs), two of them did not.



DANNY'S PROTOCOL FOR NATURAL TRANSFORMATION (Also in “Protocols” Section of Wiki)

- The protocol that follows was designed to screen for recombination events, so it will be useful for the FLP construct transformation.
- For preparation of A+ medium (containing 1.0 g/L of NaNO3), refer to Steven et al. (11)
- When attempting mutagenesis of genes encoding proteins involved in photosynthesis, glycerol is frequently included in the A+ medium to provide an alternative carbon source to CO2 (see Note 9). Currently, most mutagenesis on Synechococcus sp. PCC 7002 involves the photosynthetic apparatus, therefore the protocol given here includes 10 mM glycerol in the A+ medium.
- Synechococcus sp. PCC 7002 grows optimally at 38°C; however, plates with solid medium are most conveniently incubated at, or slightly above, room temperature (typically around 30°C). It is recommended to use pipets and pipet tips with an aerosol barrier when handling liquid cultures, sterile media, and gas mixtures to prevent contamination. Strains may be frozen and stored as described in Note 16. 1.

1. Prepare an exponentially growing culture of Synechococcus sp. PCC 7002 at 38°C in liquid A+ medium containing 10 mM glycerol and bubbled with sterile air supplemented with 1% CO2.
2. Harvest the cells and resuspend them in fresh medium to an OD730 nm of 2 to 3.
3. Mix 1 to 5 µg of DNA and 0.8 mL of culture and incubate under strong illumination (about 250 µE m2 s1) at 38°C for 5 h while gently bubbling with sterile air supplemented with 1% CO2. (Keep the volume of the added DNA solution less than one-tenth of the culture volume.)
4. Spread the cell suspension over a plate with solid A+ medium containing 10 mM glycerol and allow the cells to grow under moderate illumination (about 150 µE m2 s1) at 30°C for about 3 d until a thin lawn of growth is visible.
5. Overlay the plate with sterile, melted 0.8% (w/v) agar in water containing antibiotic (not warmer than about 50°C) over the cells. Use about 3 mL per plate (diameter 9 cm) containing 40 to 50 mL of solid medium. (Adjust the amount of antibiotic added such that the final concentration is calculated for the entire volume of solid medium in the plate.)
6. Incubate the plate under the same conditions as in step 3 until single colonies appear (at least 2 to 3 wk).
7. Transfer several colonies on fresh solid, A+ medium containing 10 mM glycerol and the appropriate antibiotic and incubate again. Restreaking should be repeated until a homozygous isolate is obtained (two to three restreakings from a single colony is usually sufficient unless the gene product is required for viability or strongly selected for under the growth conditions employed).
8. For each clonal isolate, streak one colony in a small area (about 1 cm2) on a fresh plate and incubate until a thick patch of cells appear. Use this patch of cells for verification of allele segregation.

Danny set up plates of cyanobacteria and placed three into the shaking incubator and three into the carbon dioxide incubator to test the effect of carbon dioxide on growth.

Holly prepared electrocompetent CIAT rhizobium cells and then attempted to electroporate [pKT230] into them. Holly also ran a LIC cassette incorporation test.

- Preparing electrocompetent cells
  - Purpose: more efficient electroporation in rhizo
  - Procedure (may be incomplete; refer to Lionel)
    a. Grow 500 mL of cultures to mid-log
    b. In the 4C cold room, store saturated ice buckets of water and glycerol (to make saturated ice bucket, fill about half the bucket with ice, add water to pack the ice more tightly, then add more ice on top; ice should surround the bottle of water or glycerol)
    c. Transfer 250 mL of mid-log cultures to large centrifuge tubes
    d. Spin down for 15 min at 5000 rpm
    e. Wash 2x with 250 mL water, 1x with 10% glycerol (100 mL)
       i. You wash with glycerol because you will be storing this in -80C; cells have less surface tension when stored in glycerol so won't crack when freeze-thawed
       ii. After the first wash, resuspend and combine contents; wash 2nd time in 250 mL water
    f. After 3rd resuspension (in glycerol), centrifuge and remove supernatant
    g. Resuspend in 150 mL glycerol (? Not sure about this step)
    h. Transfer 1 mL to microcentrifuge tube
    i. Store in -80C
       - Should end up with 100+ tubes

  Electroporation of electrocompetent cells

  Purpose: transform pkt230 into CIAT

  Notes:

  - Do everything on ice
  - Add 1 uL plasmid to 20 uL electrocomp. culture
  - Use 1 mL recovery media
  - EP settings: 2.5 V for 2 mm cuvette (1.25 V for 1 mm cuvette), 200 Ohms, 250 uF

Cuvette	Time constant
CIAT [pkt230]	6.5
CIAT [pkt230]	4.0
-ve	6.5

- LIC Cassette incorporation test:
  - Purpose: to determine which E. coli colonies have successfully taken up the LIC cassette
    - If they have the insert (sacB), should not be able to grow on sucrose
    - Should be able to grow on kan
  - Procedure
    - Pick from single colonies and streak on selective plates
      - Colonies can come from plates of any dilution
      - Selective plates: 1st streak on suc, then streak on kan (~1.25 cm long streaks); one plate contains about 48 streaks from 48 diff colonies (include NC)

    - Mix the cells (use the same streaking stick as from step 1) in well plates
        - Purpose: based on the cells that give correct results in step 1, you can use the cells that grew in the corresponding well (liquid culture) for mini prepping

Lionel ran a midiprep of two forms of pKT plasmid. Lionel also ran a PCR to check the orientation of the LIC cassette after ligation.

7/29

Colin gel extracted his PCR products from yesterday, getting good yield with Lambda CI and bad yield with Anderson Weak promoter. Colin miniprepped KT230 DNA today for natural transformation and cut it with SacI. Colin ran a natural transformation protocol of his FLIP construct into 7002, both in the dark and in the light.

Danny successfully ran a gradient PCR (53 degrees C to 63 degrees C) to amplify NirA and CpcAB for the CRISPR construct. Both amplified products were extracted and gel purified, leading to yields of about 30 nanograms per microliter. Surprisingly, colder temperatures led to larger amounts of product.

Lionel ran a restriction enzyme digest of [pKT230] and the various derivatives of the plasmid he had made to test what was going on. Lionel used EcoR1, BamH1, and Bsa1. Lionel ran a miniprep of the cultures from ligation 2.

7/30

Colin plated the cells he had transformed yesterday.
A FLP construct with reporters for testing mutagenesis efficiency:

- For PCC 7002
- Basic design of new construct (upstream to downstream):
    - 1 kb homology
    - FRT site
    - kanR (from pKD4)
    - FRT site
    - psaA promoter
    - Citrine (with premature stop codon at Glu-91)
    - AmpR + terminator (with premature stop codon at Gln-86, from pETcoco-2)
    - 1 kb homology
- Synthetic RBS's upstream of the citrine and ampR genes were designed by the De Novo DNA RBS Calculator (sequence: ataaggtcaaaaagtagcagaaggaggttaaa for both, TIR = 13600)
- Colin ordered primers to build this construct

Danny checked the selective media plates of the natural transformation. Not much growth had occurred. Danny ran a Gibson assembly of the Crispr construct and ran an amplification reaction to check to see if the Gibson assembly was successful.

Holly checked the results of her antibiotic resistance assay. These are the results:

	LB Kan	TSB Kan	LB Carb	TSB Carb	LB Spec	TSB Spec
CIAT			+	+	+	+
356	+					-
370	+		+			-
371	+		?		+	+

Holly also checked the results of her electroporation two days ago. The negative control plates had lawns. This should not be the case.

7/31

Danny's last day! He checked the growth of the plates in the carbon dioxide versus no carbon dioxide experiments. While the carbon dioxide incubator plates had lawn growth, the shaking incubator plates had only very small colonies. Therefore, carbon dioxide is a major factor of cyanobacterial growth.

Steps to Accomplish:

For Crispr:

1. Finish gibson Assembling the linear construct
    i. Run a gel of amplified gibson product (All/ CG PCR tubes)
    ii. Hope everything works
    iii. Sequence it?
    iv. Trouble shoot if unsuccessful
2. Gibson assemble/ LIC the construct into pKT230
3. Transform the pKT230-CRISPR into DH5Alpha
4. Grow up this strain
5. Miniprep the Strain once we've nailed down a transformation protocol
6. Transform the pKT230-CRISPR plasmid into 7002 (The 7002 needs to be grown in Nitrateless A+. This will slow down growth but still allow it to survive.)
7. Test the sensitivity of this new strain of 7002 in Urea only and Urea and Nickel Sulfate solutions using the protocol in the CRISPR subection of Cyanobacteria Projectnotes as an example.
8. If we obtain successful, healthy growth in Urea and Nickel Sulfate, we have (hopefully) proved that CRISPR systems work in 7002.

For Promoters:

1. Run a gel of the Lambda CI promoter and gel extract it
2. Gibson assemble the Lambda CI promter and citrine together
3. Run a gel with all of the gibson assembled Promoter-citrine constructs (Lambda CI, pBAD, Anderson/TetR complex).
4. Check if gibson assemblies were successful
5. Gibson assemble or LIC into pKT230
6. Transform into Cyano and check Fluorescence

For Transformation

1. Continue to monitor growth of plates inside the CO2 incubator
2. After 9 Days, (Friday is the fourth day), wash one plate of Streaked cells, collect cells, and run a Live/Dead Stain to assess health/survival of cells. For both Trial/ Circ (Circularized)/ and Control.
3. The plates are labelled Trial for linearized DNA, Circ for Circulairzed DNA, and Cont for control. The plates are also labelled Str (1, 2, or 3) for streaked cells, and wash (50 or all) for plates coated in cells from plate washes. Those labelled 50 were washed with 50 microliters. Those labelled all were washed with the remaining liquid, often about 1.5 mL.
4. Possible reasons for failure: Incubator Heat issues. Washing the plates with A+ without Kan. Failure in protocol. Failure in proper streaking technique.
5. Also, the Control Wash All plate was contaminated with a Kim Wipe. We can determine a negative result from it (no cyano was able to grow), but any positive result (growth) may be contamination and would require a re running of the trial.

8/1

Colin checked the OD650/OD730 of UTEX and 7002 to see which produced more Chlorophyll. It appeared that UTEX did.

Colin continued to check his growth assay for different medias. With B12 corrected, A+ has shown growth.

8/2

Colin checked growth assay/ natural transformation. Natural transformation seemed to be growing!

WEEK 10 (8/3 to 8/9, 2015)

Colin - 8/2/15

NATURAL TRANSFORMATION OF PCC 7002

- Liquid Cultures:
  - No change in the growth of the liquid cultures inoculated on 7/30 (cultures on no antibiotic are very green, cultures in media with antibiotic show no growth)
  - Cultures inoculated from antibiotic-free media into selective media yesterday seem to be growing!

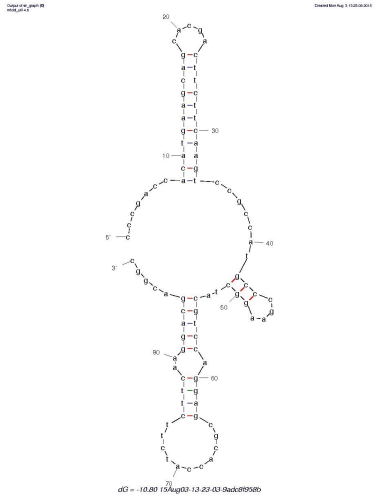
Sample ID	OD730
1 - Kan	0.22
3 - Kan	0.24
5 - Kan	0.44
7 - Kan	0.34
9 - Carb	0.32
11 - Carb	0.31

  - Will read ODs again tomorrow to verify extent of growth

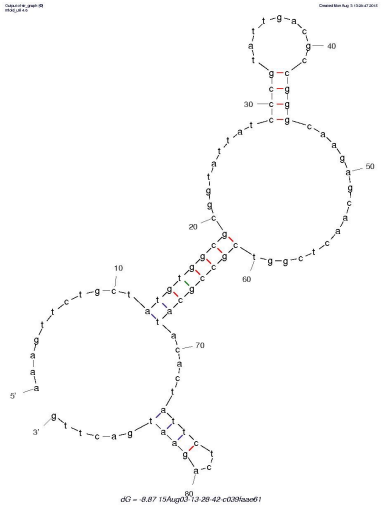
Colin - 8/3/15

FOLDING CHARACTERISTICS OF OLIGOS IN FLP-REPORTER CONSTRUCT

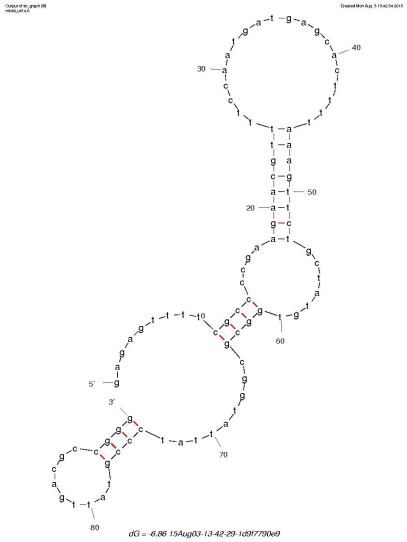
- At Glu-91 on citrine:
  - 60% GC content
  - Structure at -10.8 kcal/mol:



- Alternative point mutations on citrine:
  - Tyr-85: -9.99 kcal/mol
  - Trp-58 (TGG to TAG): -8.34 kcal/mol
  - Glu-7 (49% GC, GAG to TAG): -8.06 kcal/mol
    - This one will also eliminate a fragment for assembly, since we can incorporate the mutation into the forward primer for citrine. Let's go with this one.
- At Gln-86 on AmpR:
  - 51% GC content
  - Structure at -8.87 kcal/mol:



  - Lys-71 (AAA to TAA) might be better for ampR cassette (49% GC, -6.34 kcal/mol):



- Structures and free energies determined using mfold server

NATURAL TRANSFORMATION OF PCC 7002

- Growth of cultures inoculated into selective media on 8/1:

Sample ID	OD730
1 - Kan	0.21
3 - Kan	0.20
5 - Kan	0.26
7 - Kan	0.22
9 - Carb	0.34
11 - Carb	0.12

  - No growth in any of these samples. In case the lack of growth is due to nutrient loss, 20 µg/l vitamin B12 was added to each of these samples.
- Status of liquid cultures inoculated on first day (7/30):

Sample	DNA	Incubation Condition	Selection	Growth?
1	FLP Construct	Light	None	Y
2	FLP Construct	Light	Kan	Y
3	FLP Construct	Dark	None	Y
4	FLP Construct	Dark	Kan	Y
5	pKT230	Light	None	Y
6	pKT230	Light	Kan	N
7	pKT230	Dark	None	Y
8	pKT230	Dark	Kan	N
9	pETcoco-2	Light	None	Y
10	pETcoco-2	Light	Carb	N
11	pETcoco-2	Dark	None	Y
12	pETcoco-2	Dark	Carb	N
13	n/a	Light	None	Y
14	n/a	Light	Kan	N
15	n/a	Light	Carb	N
16	n/a	Dark	None	Y
17	n/a	Dark	Kan	N
18	n/a	Dark	Carb	N

- Cells transformed with the FLP construct seem to be growing on selective media!
  - Could be due to kanamycin failure
- 100 µl of samples with growth in this group was inoculated into 4 ml of A+ medium with 20 µg/l B12 and 100 µg/ml of either kan or carb, depending on the expected outcome:

Sample	Conditions	Initial OD730
1	FLP, light	0.13
2	FLP, light, kan	0.010
3	FLP, dark	0.14
4	FLP, dark, kan	0.020
5	pKT230, light	0.020
7	pKT230, dark	0.15
9	pETcoco-2, light	0.13
11	pETcoco-2, dark	0.17

Colin - 8/4/15

NATURAL TRANSFORMATION OF PCC 7002

- After 5 days, cultures plated on non-selective A+ media showed lawn growth. Selective media was introduced to these cultures in two ways:
  - 500 µl of A+ media was used to wash some of the cells off the plate, and this cell suspension was plated on selective media
  - 5 ml of 0.8 % A+ agar (with B12 and thiosulfate) was overlayed on top of the plates with lawn growth. This is the method outlined in the transformation protocol which Danny found.

PCR OF FLP-TRANSFORMED CELL POPULATIONS

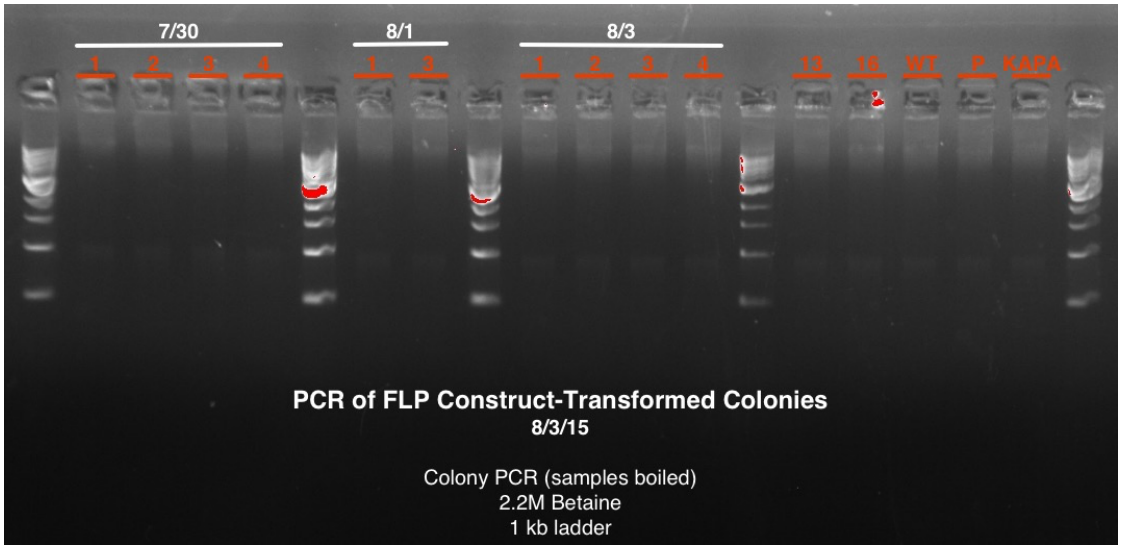
- Use primers 378 and 383 for all amplicons
- Predicted size of WT fragment (mutS intact): 4600 bp
- Predicted size of KO fragment (mutS replaced by FRT-kanR-FRT): 3000 bp
- Samples:

Sample	KAPA HiFi	F_Primer (0.5 µl)	R_Primer (0.5 µl)	Template (1.0 µl)	2.2M Betaine	Notes	Name on Gel
1	20 µl	378	383	1 - 7/30	18 µl	Light, no selection, inoculation on 7/30	1
2	20 µl	378	383	2 - 7/30	18 µl	Light, selection, inoculation on 7/30	2
3	20 µl	378	383	3 - 7/30	18 µl	Dark, no selection, inoculation on 7/30	3
4	20 µl	378	383	4 - 7/30	18 µl	Dark, selection, inoculation on 7/30	4
5	20 µl	378	383	1 - 8/1	18 µl	Light, selection, inoculation on 8/1	1
6	20 µl	378	383	3 - 8/1	18 µl	Dark, selection, inoculation on 8/1	3
7	20 µl	378	383	1 - 8/3	18 µl	Light, selection, inoculation on 8/3	1
8	20 µl	378	383	2 - 8/3	18 µl	Light, selection, inoculation on 8/3	2
9	20 µl	378	383	3 - 8/3	18 µl	Dark, selection, inoculation on 8/3	3
10	20 µl	378	383	4 - 8/3	18 µl	Dark, selection, inoculation on 8/3	4

11	20 µl	378	383	13 - 7/30	18 µl	WT control (light)	13
12	20 µl	378	383	16 - 7/30	18 µl	WT control (dark)	16
13	20 µl	378	383	7002 in A+	18 µl	WT control (culture from Sample 1 of current growth assay)	WT
14	20 µl	378	383	n/a	19 µl	Control	P
15	20 µl	n/a	n/a	n/a	20 µl	Control	KAPA

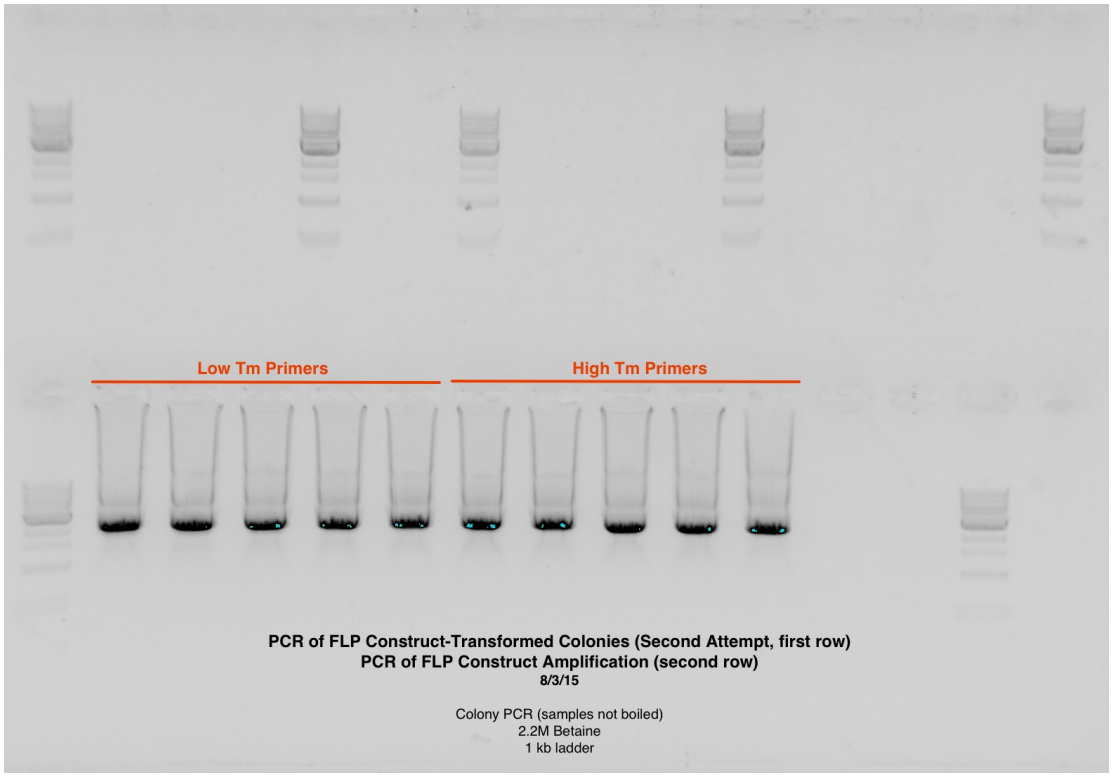
- Protocol:
  a. 95 ˚C for 5 min
  b. 98 ˚C for 20 sec
  c. 60 ˚C for 15 sec
  d. 72 ˚C for 4.5 min
  e. 32 cycles of b to d
  f. 72 ˚C for 5 min

- Gel:
  - 1 % EtBr, 120 V, 400 mA, 35 mins



  - Something seems to have gone wrong with the PCR since there are no bands anywhere.
  - New plan for second PCR:
    - Don't boil templates
    - Extension step for 5 min

    - Make fresh primer stocks
    - Annealing step at 59 ˚C
- Gel of second PCR:
    - 1 % Sybr, 120 V, 400 mA, 30 mins



    - FLP construct amplified successfully. Good to know that PCR products can last about a week in the -20 and still give good yields on gels.
    - Same results as first attempts for amplification of mutS region in cells. Nothing amplified, including any of the controls. So it would be wrong to conclude that the construct recombined in the wrong part of the genome. It's still probably a problem with the PCR protocol. Bad KAPA? Too short annealing time? Will try again tomorrow.

Colin - 8/5/15

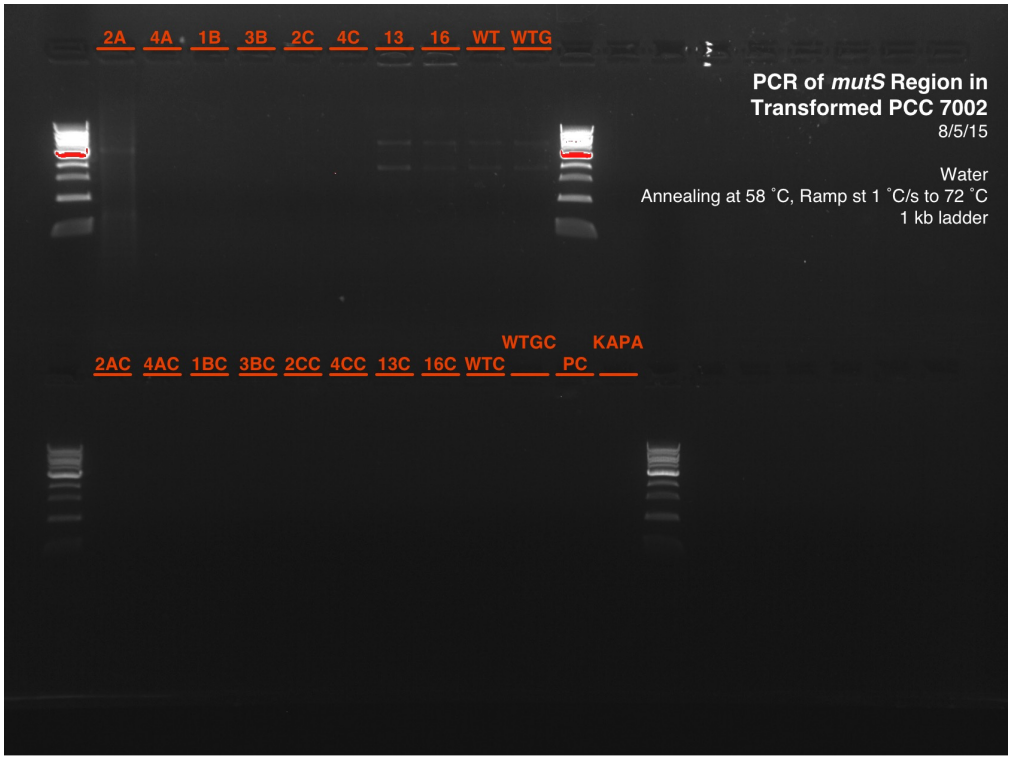
PCR VERIFICATION OF FLP CONSTRUCT RECOMBINATION – THIRD ATTEMPT

- Samples:

Sample	KAPA HiFi	F_Primer (0.5 µl)	R_Primer (0.5 µl)	Template (1.0 µl)	Nuc-Free Water	Notes
2A	20 µl	378	383	2 - 7/30	18 µl	Light, selection, inoculation on 7/30
2AC	20 µl	378	383		18 µl	
4A	20 µl	378	383	4 - 7/30	18 µl	Dark, selection, inoculation on 7/30
4AC	20 µl	378	383		18 µl	
1B	20 µl	378	383	1 - 8/1	18 µl	Light, selection, inoculation on 8/1
1BC	20 µl	378	383		18 µl	
3B	20 µl	378	383	3 - 8/1	18 µl	Dark, selection, inoculation on 8/1
3BC	20 µl	378	383		18 µl	
2C	20 µl	378	383	2 - 8/3	18 µl	Light, selection, inoculation on 8/3
2CC	20 µl	378	383		18 µl	
4C	20 µl	378	383	4 - 8/3	18 µl	Dark, selection, inoculation on 8/3
4CC	20 µl	378	383		18 µl	
13	20 µl	378	383	13 - 7/30	18 µl	WT control (light)
13C	20 µl	378	383		18 µl	
16	20 µl	378	383	16 - 7/30	18 µl	WT control (dark)
16C	20 µl	378	383		18 µl	
WT	20 µl	378	383	7002 in A+	18 µl	WT control (culture from Sample 1 of current growth assay)
WTC	20 µl	378	383		18 µl	
WTG	20 µl	378	383	7002 in	18 µl	WT control (culture

				A+/10 mM glycerol		from Sample 1 of current growth assay)
WTGC	20 µl	378	383		18 µl	
PC	20 µl	378	383	n/a	19 µl	Primer Control
KAPA	20 µl	n/a	n/a	n/a	20 µl	KAPA Control

- Notes on procedure:
  - Samples were not boiled, but were taken straight from cultures
  - Nuc-free water was used instead of 2.2M betaine
  - Fresh KAPA mix was used
- PCR protocol (saved as "FLPGRAD" in PCR 5-7 block):
  - 95 ˚C for 5 min
  - 98 ˚C for 15 sec
  - 58 ˚C for 15 sec
  - 72 ˚C for 5 min
    1. The ramp rate up to this step from 58 ˚C is 1 ˚C/s
  - Repeat steps b-d 34x
  - 72 ˚C for 5 min
  - 4 ˚C forever
- Gel of PCR Products
  - 1 %, EtBr, 120 V, 400 mA, 30 mins



    - WAT. The four wild-type controls (upper right) have two bands each in the came location. This is unexpected (there should only be one band at 4.6 kb) but at least it indicates that something happened to the transformed cells' genome at the region where the primers amplified. It's possible that the primers amplified two different regions within the cell's genome, resulting in two different bands. In that case, this is a really inefficient PCR. The 2A experimental sample has some weird bands and a smear, and is clearly different from the other experimental samples. Not sure how to explain this.
- Troubleshooting
    - Ran a BLAST search of the 1 kb homology regions and the primers used to amplify region
        - There appear to be no other regions where the primers could anneal reliably and confound results (highest Tm of a primer subsequence was 51 ˚C)
        - There also appears to be no somewhat similar sequence (determined using BLASTN) for either 1 kb homology arm, so it seems unlikely that the construct could have recombined elsewhere. The gel results would reinforce this, since there is an obvious difference between the transformed and WT genomes.
    - It might be helpful to run the annealing for a bit longer and/or at a higher temperature (say, start at 60 ˚C and ramp up to 72 really slowly)
    - Could the FLP construct itself be altering the sequence surrounding mutS? Don't know how this would work, but maybe its possible. In that case, it would be best to run PCRs with primers closer to the actual gene. The primers ordered yesterday could tell us this.

GEL EXTRACTION OF AMPLIFIED FLP CONSTRUCTS

- Each sample is in 47 µl of nuc-free water

Sample	**Concentration (ng/µl)**	**260/280**
1	30.5	1.74
2	30.3	1.74
3	28.2	1.72
4	38.2	1.69
5	43.6	1.73
6	43.6	1.71
7	48.5	1.72
8	37.6	1.72
9	33.6	1.70
10	40.3	1.69

- So total yield is about 16 µg of construct… Good for a lot of transformations.

- Combined all samples into one Eppi tube; final concentration is 37.4 ng/µl (27.7 µl of solution is needed to have 1 µg of FLP construct).

DETERMINING ONCE AND FOR ALL IF THE TRANSFORMED PCC 7002 COLONIES CAN GROW IN KAN MEDIA

- 200 µl of each of the following cultures was inoculated into 4 ml of A+ medium with 20 µg/l B12 and 200 µg/ml of kanamycin. If growth is observed in any of the first 6 samples, we can confidently say that the transformation was successful.

Sample	Notes
2A	Incubation in light, inoculation into 100 µg/ml kan on 7/30
4A	Incubation in dark, inoculation into 100 µg/ml kan on 7/30
1B	Incubation in light, inoculation into 100 µg/ml kan on 8/1
3B	Incubation in dark, inoculation into 100 µg/ml kan on 8/1
2C	Incubation in light, inoculation from 2A into 100 µg/ml kan on 7/30
4C	Incubation in dark, inoculation from 4A into 100 µg/ml kan on 7/30
13	Incubation in light, no DNA
16	Incubation in dark, no DNA
WT	PCC 7002 in A+
WTG	PCC 7002 in A+/glycerol

COLLABORATIONS

- La Verne replied saying that they are interested in collaborating to make a handbook for iGEM teams working in non-model strains. We might have a Skype call.
- Questions to ask them via Skype:
  - Have you contacted any other teams working in non-model strains?
  - Are you successfully culturing your non-model strains?
    - How long had you anticipated this would take at the beginning of the summer?
    - What gave you trouble?
    - What does your growth setup look like?
  - We noticed that you are experimenting with LIVE/DEAD staining of cyanobacteria. How did you come across this method? How are you planning to use it? Are you planning to use it in a flow cytometer?
  - Here are the steps we believe are essential that teams should take before they can start working with non-model strains in earnest:
    - Culturing (growth, media types, incubation conditions, solid vs. liquid)
    - Selection using antibiotics or reporter molecules
    - Transformation (natural competency, chemical, electroporation, conjugation)
    - On which of these steps do you have good data? Can you think of anything else (whether you have tried it or not) that might give you or other teams trouble?
  - You going to the Jamboree?

Colin - 8/6/15

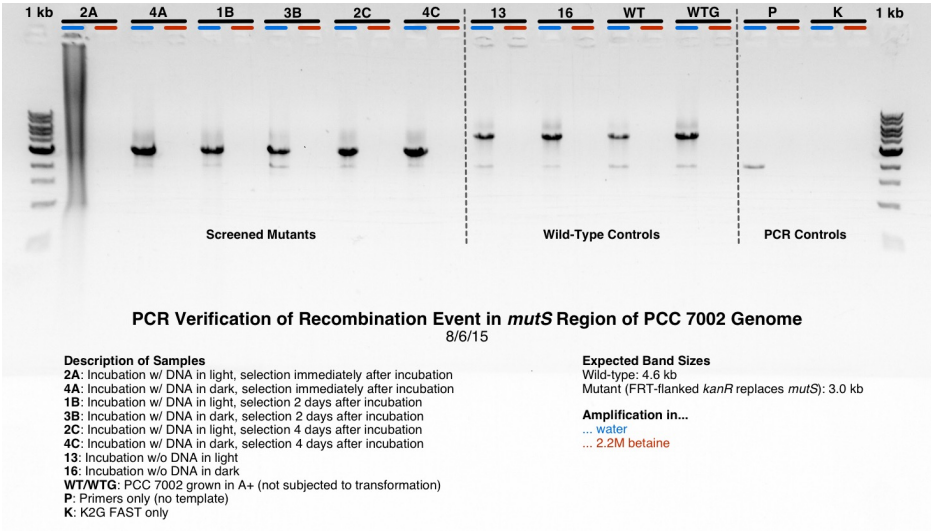
PCR VERIFICATION OF FLP CONSTRUCT RECOMBINATION – FOURTH ATTEMPT

- Samples:

Sample	K2G	F_Primer (0.5 µl)	R_Primer (0.5 µl)	Template (2.0 µl)	Nuc-Free Water OR 2.2M Betaine	Notes
2A	20 µl	378	383	2 - 7/30	18 µl	Light, selection, inoculation on 7/30
4A	20 µl	378	383	4 - 7/30	18 µl	Dark, selection, inoculation on 7/30
1B	20 µl	378	383	1 - 8/1	18 µl	Light, selection, inoculation on 8/1
3B	20 µl	378	383	3 - 8/1	18 µl	Dark, selection, inoculation on 8/1
2C	20 µl	378	383	2 - 8/3	18 µl	Light, selection, inoculation on 8/3
4C	20 µl	378	383	4 - 8/3	18 µl	Dark, selection,

						inoculation on 8/3
13	20 µl	378	383	13 - 7/30	18 µl	WT control (light)
16	20 µl	378	383	16 - 7/30	18 µl	WT control (dark)
WT	20 µl	378	383	7002 in A+	18 µl	WT control (culture from Sample 1 of current growth assay)
WTG	20 µl	378	383	7002 in A+/10 mM glycerol	18 µl	WT control (culture from Sample 1 of current growth assay)
PC	20 µl	378	383	n/a	19 µl	Primer Control
KAPA	20 µl	n/a	n/a	n/a	20 µl	KAPA Control

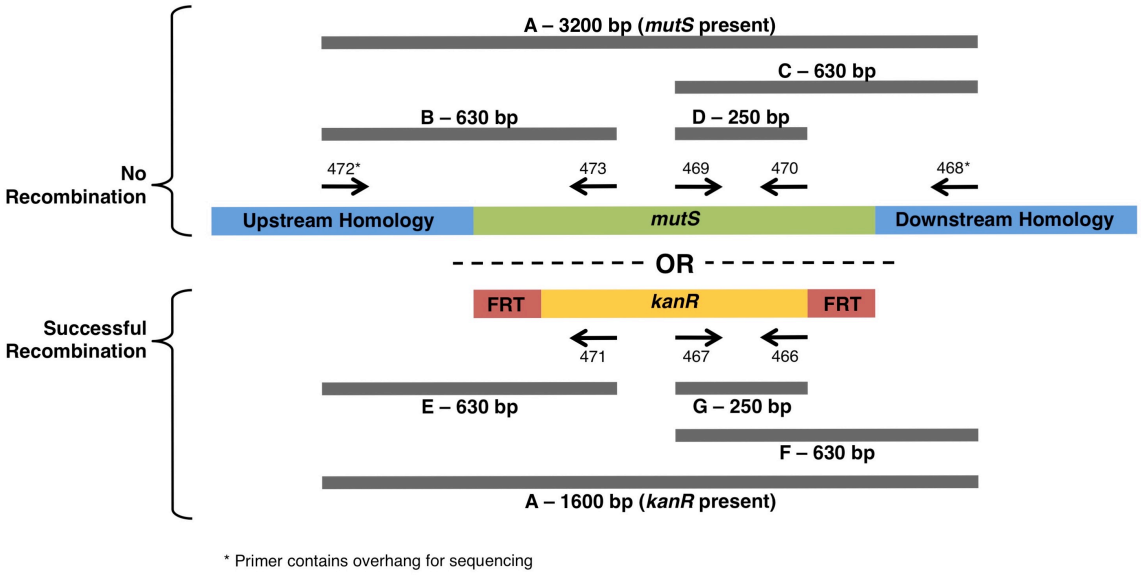
- K2G was used instead of KAPA HiFi
- Templates were not boiled
- Reactions in nuc-free water and 2.2M betaine were prepared and run in parallel
- PCR protocol: FLPGRAD, 34 cycles
- The 5 min extension time is probably way too long for the K2G polymersase; KAPA recommends extension times of 15 sec/kb if the target amplicon is larger than 1 kb. So 1:15 would have been fine. Possible troubleshoot for later.
    - KAPA also recommends not annealing below 60 ˚C with K2G… Something else to modify next time.
- Gel of PCR products:
    - 0.85 %, EtBr, 1 kb ladder, 120 V, 400 mA, 35 mins



- This serves as really strong evidence that the construct successfully recombined in the experimental samples! The cells look like they have a relatively clean genotype; there don't seem to be any mutS regions that were amplified in the experimental cells (there is a little bit of ghosting above the bands, but this also appears above the wild-type sample bands).

PCR VERIFICATION AND SEQUENCING OF *mutS* RECOMBINATION: ASSAY DESIGN

- Basic design:



- 7 pairs of primers will be used, and 8 amplicons are possible (primer set A could yield two different amplicons depending on the genotype):

Amplicon ID	F_Primer	R_Primer	Expected Size (bp)	Fragment Description
A	472	468	3200/1600	mutS + context AND/OR kanR + context
B	472	473	630	mutS upstream
C	469	468	630	mutS downstream
D	469	470	250	mutS internal
E	472	473	630	kanR upstream
F	467	468	630	kanR downstream
G	467	466	250	kanR internal

- Right now, all primers except 471, 472, and 473 are on hand

- PCR with currently available primers:

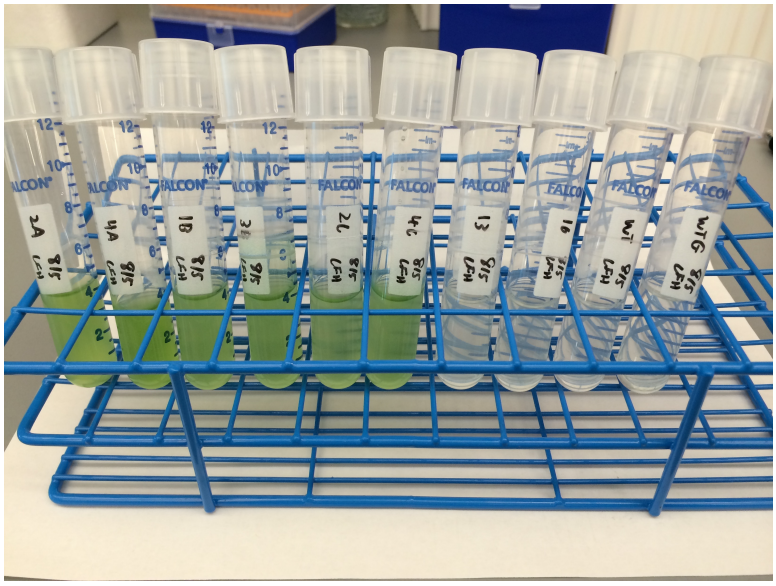
Well Number	Colony	Sample/Target Amplicon	K2G	F_Primer	R_Primer	Template (0.5 µl)	Nuc-Free Water (5.5 µl)	Notes
1	2A	C	6 µl	469	468	2A		
2		D	6 µl	469	470	2A		
3		F	6 µl	467	468	2A		
4		G	6 µl	467	466	2A		
5	4A	C	6 µl	469	468	4A		
6		D	6 µl	469	470	4A		
7		F	6 µl	467	468	4A		
8		G	6 µl	467	466	4A		
9	1B	C	6 µl	469	468	1B		
10		D	6 µl	469	470	1B		
11		F	6 µl	467	468	1B		
12		G	6 µl	467	466	1B		
13	3B	C	6 µl	469	468	3B		
14		D	6 µl	469	470	3B		
15		F	6 µl	467	468	3B		
16		G	6 µl	467	466	3B		
17	2C	C	6 µl	469	468	2C		
18		D	6 µl	469	470	2C		
19		F	6 µl	467	468	2C		
20		G	6 µl	467	466	2C		
21	4C	C	6 µl	469	468	4C		
22		D	6 µl	469	470	4C		
23		F	6 µl	467	468	4C		
24		G	6 µl	467	466	4C		
25	13	C	6 µl	469	468	13		Light control
26		D	6 µl	469	470	13		
27		F	6 µl	467	468	13		
28		G	6 µl	467	466	13		

29	16	C	6 µl	469	468	16		Dark control
30		D	6 µl	469	470	16		
31		F	6 µl	467	468	16		
32		G	6 µl	467	466	16		
33	WT	C	6 µl	469	468	WT		Untransformed control
34		D	6 µl	469	470	WT		
35		F	6 µl	467	468	WT		
36		G	6 µl	467	466	WT		
37	WTG	C	6 µl	469	468	WTG		Glycerol culture control
38		D	6 µl	469	470	WTG		
39		F	6 µl	467	468	WTG		
40		G	6 µl	467	466	WTG		
41	P	C	6 µl	469	468	n/a		Primers control
42		D	6 µl	469	470	n/a		
43		F	6 µl	467	468	n/a		
44		G	6 µl	467	466	n/a		
45	K	n/a	6 µl	n/a	n/a	n/a		KAPA control
46		n/a	6 µl	n/a	n/a	n/a		
47		n/a	6 µl	n/a	n/a	n/a		
48		n/a	6 µl	n/a	n/a	n/a		

- All C/D/F/G amplicons will be set up in the same tube strip. C/D will be overlayed in one row of the gel, and F/G will be overlayed in the other row.
- It might also be a good idea to re-run the *mutS* region PCR with the exact same samples that will be used for this reaction.

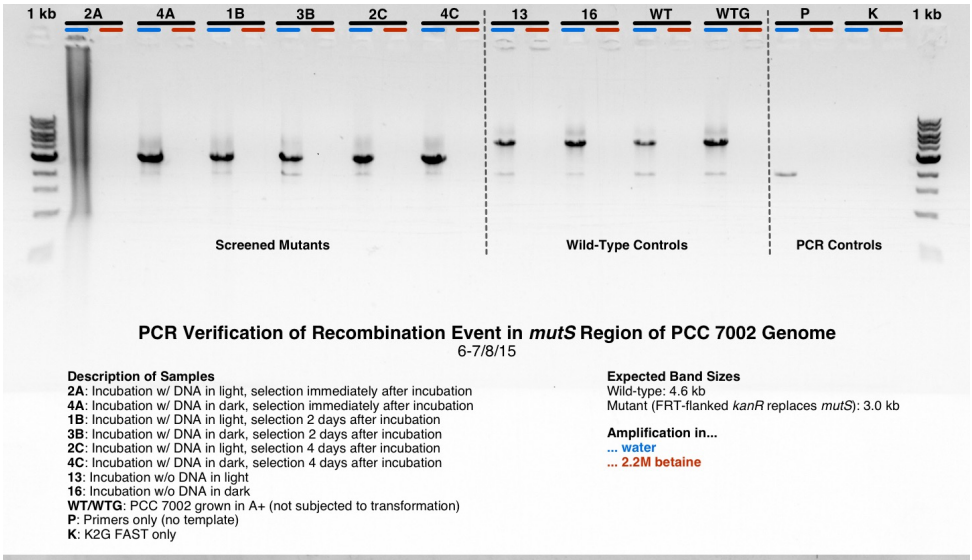
Colin - 8/7/15

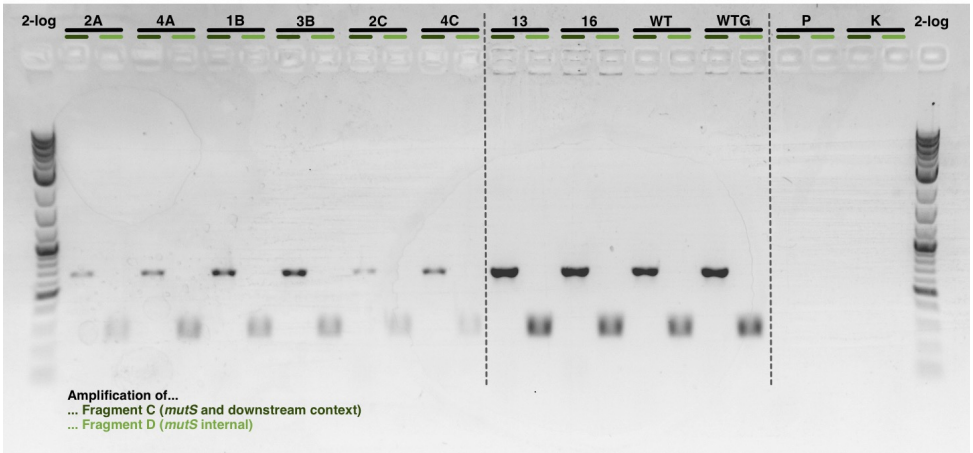
PCR VERIFICATION AND SEQUENCING OF *mutS* RECOMBINATION

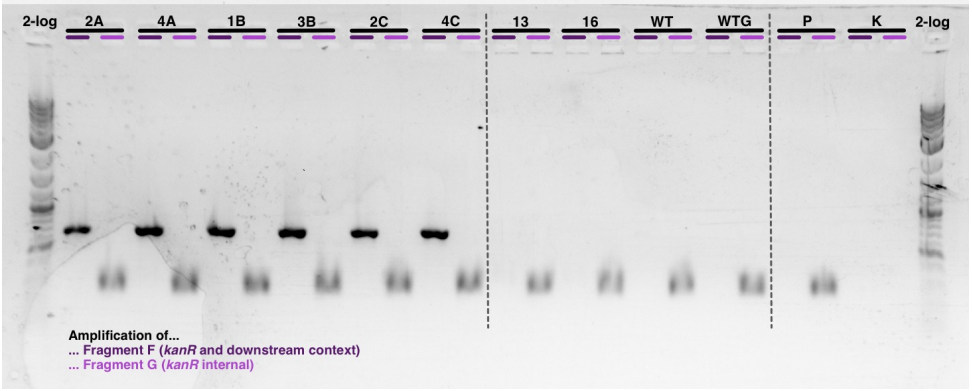


- Ran the small region PCR described on 8/6 (48-well experiment)
    - Protocol: FLPGRAD (15 sec extension time, 58 ˚C annealing temp, in water)
- Re-ran the whole region PCR that was attempted yesterday with the freshly grown templates of experimental samples in Kan, so that the template is the same for both this PCR and the small region PCR
- Layout of gel:
    - First Row: Target amplicons C and D (*mutS* verification) overlayed next to one another
    - Second Row: Whole region amplification (rerun from yesterday's PCR with the same templates used as the subsection PCR)
    - Third Row: Target amplicons F and G (*kanR* verification) overlayed next to one another

- Gel:
  - 350 ml, EtBr, 120 V, 400 mA, 50 mins







  - Definitely looks like we have some partial mutants! The genotypes don't appear to be entirely homozygous, but based on band strengths of the mutS screen (green), some of the mutS copies have been knocked out. It's wierd that bands show up in the controls of the kanR screen (purple), but this might just be primer dimer (second to last lane).

THE 12 µl PCR: MIX FOR 12 WELLS

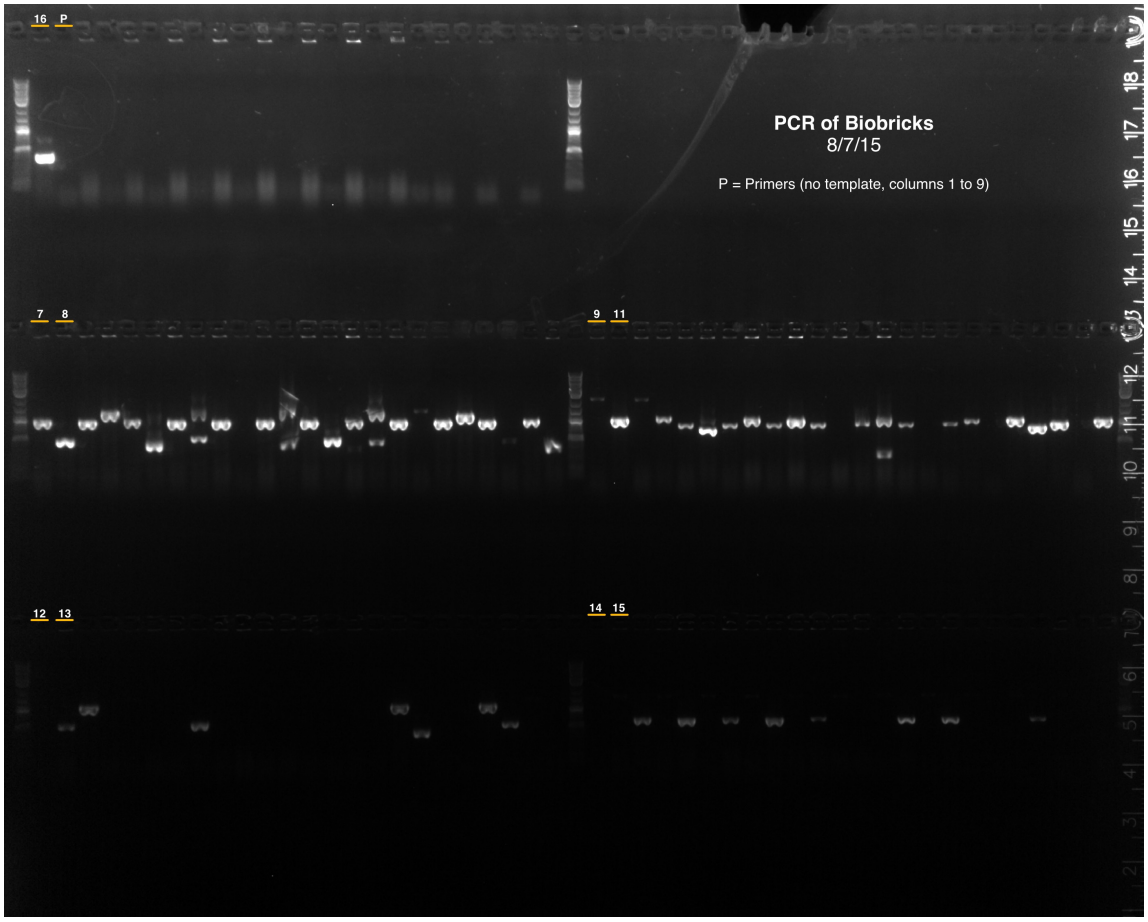
- 72 µl ReadyMix (HiFi or K2G)
- 1.8 µl working forward primer stock (20 µM)
- 1.8 µl working reverse primer stock (20 µM)
- 62.4 µl (variable) of nuc-free water or 2.2M betaine
- 6 µl of template (or 0.5 µl of template into each well, after aliquoting 11.5 µl of master mix into individual PCR tubes)

ARIEL'S PCR

- Master mix for 12 wells:
  - 72 µl K2G
  - 60 µl nuc-free water
  - 2.64 µl forward primer
  - 2.64 µl reverse primer
  - 0.7 µl template into each well
- 96-well PCR plate setup:

Row	Forward Primer	Reverse Primer	Template
A	pSB1C3_M13seq_F	399	7
B	pSB1C3_M13seq_F	401	8
C	pSB1C3_M13seq_F	403	9
D	pSB1C3_M13seq_F	407	11
E	pSB1C3_M13seq_F	409	12
F	pSB1C3_M13seq_F	411	13
G	pSB1C3_M13seq_F	413	14
H	pSB1C3_M13seq_F	415	15
A2	pSB1C3_M13seq_F	417	16
B2 (columns 1-9)	pSB1C3_M13seq_F	(Primers only)	n/a

- Gel (500 ml, EtBr, 175 V, 400 mA, 25 mins):



Colin - 8/8/15

PCC 7002 CULTURES

- Made glycerol stocks of:
  - PCC 7002 (sample from growth assay)
  - UTEX 2973 (sample from growth assay)
  - PCC7002.2A, .4A, .1B, .2C, .4C (from FLP construct transformation)
  - iGEM database ID's 123-130
  - Glycerol stock composition: 350 µl growth media + 150 µl 80% glycerol
- Made large 20 ml cultures of PCC7002.2C and PCC7002.4C, since these seem to have the cleanest, most mutS-free genotypes. Will do dilutions and plate for single colonies tomorrow
- Also inoculated 10 µl of samples 5, 7, 9, and 11 from transformation into A+/kan (for 5 and 7) and A+/carb (for 9 and 11)

Colin - 8/9/15

PLATING TRANSFORMANTS

- Poured A+ and A+/Kan 200 plates
- Will plate serial dilutions of samples 2C and 4C
- It would be a really good idea to redo this transformation later on and plate the liquid cultures on selective and nonselective agar to calculate transformation/recombination efficiency, but the only samples of 2C and 4C that are on hand are already in selective media. This would be a first step to figuring out how well 7002 does MAGE by itself. If we incorporate Cas9 into the 7002 genome, we could run both transformations in parallel to get an idea of how insert length affects recombination efficiency.
- Plated tenfold serial dilutions (10^{-1} to 10^{-6}) on A+/Kan200 plates

BOARD MEETING

- Send an email out to wetlab team asking people to compile their lab notebook summaries
  - Pester pester pester
- Documentary
  - Danny has started contacting people
  - Need to setup a skype meeting – Alex will handle this
- Modeling
  - Joe will work on polymerase mutation predictor w/ GUI
  - I will look into cyano growth curve model
- T-Shirt
  - Let's do it through CustomInk
  - I'll look into doing this
  - Yamini will send me a list of sponsors
- Wiki Design
  - Change "outreach" to "human practices"

Colin - 8/9/15

WRAPPING UP EXPERIMENTS

- Wednesday-Friday 8/12 to 8/14: Check on serial dilution plates for single colonies. If plates look grown up and single colonies are apparent, count the number of SC's on each plate. Place plates in fridge for storage until I get back.
- Finished designing primers for FLP-reporter construct